SYNTHESIS OF CAROTENOID PIGMENTS IN DETACHED BEAN LEAVES

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 609

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Introduction

Studies on the mechanism of the synthesis of carotenoid pigments in higher plants have been confined almost entirely to experiments involving the observation of the development of these pigments in ripening fruit and in etiolated leaves. In addition, other studies have been made of the synthesis of pigments in lower plants, particularly the fungi. Factors which have deterred work on the green leaf are (*a*) the slow rate of synthesis observed, so that in experiments of short duration the increase in pigment is often within the variability of the pigment content of different leaves, and (*b*) the remarkable constancy of the ratios of the plastid pigments despite great environmental stresses.

The observation by WILLSTÄTTER and MIEG (**23**) of the structural relationship of carotene and phytol—the alcohol of chlorophyll—to the terpenes has led to the hypothesis that carotenoids may be derived from chlorophyll or that chlorophyll may be synthesized from carotenoids. Thus, RUDOLPH (**15**) observed an increase in chlorophylls concomitant with a decrease of carotenoids when etiolated bean seedlings were exposed to red light. STRAIN (**18**), working with etiolated barley seedlings, found, however, that both chlorophyll and carotene increased upon exposure to red light and that this was accompanied by a slow increase in carotenols. KUHN and GRUNDMANN (**9**) selected the ripening fruit of tomato to test the hypothesis; they found that the increase in lycopene was much too large to be accounted for by the phytol initially present.

This concept—the chlorophyll origin of carotenoids—has the further disadvantage that it cannot be made to extend to nonchlorophyllous plants. On the basis of the pigment content of mutant strains of the yeast *Rhodotorula*, BONNER *et al.* (**4**) suggested that phytofluene was the precursor of torulene, beta-carotene, and gamma-carotene. Since phytofluene is itself a polyene (**25**), these data do not suggest what compounds may act as precursors at an earlier stage of synthesis. A discussion of possible reactions leading to the formation of isoprene and related compounds was given by BONNER and GALSTON (**3**).

Many studies have been made on the effect of light quality and quantity on the development of carotenoids. Agreement is now fairly general that etiolated plants contain some carotene and a rather large amount of carotenol (**16**). EULER and HELLSTROM (**5**) have found that light accelerates the development of the carotenoids. It has been shown by BANDURSKI (**1**) in a nonchlorophyllous plant, *Phycomyces blakesleeanus*, that the quantity of carotene formed in darkness is only a small fraction of that formed in light and that an intensity as low as 50 foot-candles is sufficient to permit maxi-

mum development of pigment. VAN DEVENTER has shown that only the spectral region between 366 and 510 mμ is effective in stimulating development of carotenoid pigments in *Neurospora*. This work was discussed and confirmed by HAXO (**7**).

Discordant results have been obtained on the effect of blue and red light on the rate of development of the carotenoids in etiolated plants (**15, 19**). If the criterion used is the total amount of pigment formed per plant when the plants are grown under light of equal energy but different color, it is found that most carotene is formed in green light and most carotenol in red light (**22**).

In the present study most of the data have been obtained from observations on the synthesis of carotenoid pigment in cultures of bean leaves. The word "culture," as used here, has the restricted meaning indicated by the following quotation from YARWOOD (**24**): "the maintaining of leaves in a living condition for various periods after detachment from the plant of which they were once a part." The aims of the study were twofold: (*a*) to develop an experimental method sufficiently precise to enable the determination of pigment changes in green leaves over short periods of time and (*b*) to ascertain to what extent synthesis of carotenoid pigments could be disentangled from photosynthesis.

Material and methods

With the exception of two experiments performed during the early fall of 1947 the data here reported were obtained using the cordate leaves of Red Kidney bean grown in the greenhouses of the University of Chicago during the period from June, 1948, to January, 1949. The plants were allowed to grow until approximately 24 hours after the cordate leaves had emerged from between the cotyledons. By this time these leaves were one-fifth to one-fourth of their final size and had already lost the crumpled appearance which is characteristic of them for the first few hours after emergence. The tops of the plants were picked off above the cotyledonary node and placed in a moist chamber. They were then stored in a dark cupboard at ambient room temperature. The plants were picked about 6:00 P.M., and the experiment was usually begun at 3:00 P.M. the following day so that the leaves were in the dark for about 20 hours.

The samples were prepared by removing the midrib from each of the two cordate leaves of a plant and putting a half-leaf into each of four samples. Fifteen plants were used per set so that there resulted four samples, each containing fifteen half-leaves. The samples were then weighed, and one of them, the control, wrapped in cheesecloth, was blanched for 2 minutes in boiling water and stored at −20° C. until analyzed. (In a preliminary experiment involving the analysis of eleven pairs of samples the mean difference between matched samples was 1.5 ± 0.6 μgm. of carotene per gram fresh weight of leaves, or $0.8 \pm 0.5\%$ of the carotene content; thus, following the analysis of one of the four samples the initial carotene content of the remaining samples was known with a high degree of precision.) Each of the remaining three samples was then carefully floated on 50 ml. of nutrient solution contained in a 150-mm. Pyrex Petri dish half and placed in the incubation chamber. The nutrient employed was a three-salt solution consisting of KH_2PO_4 (0.0167 *M*), $Ca(NO_3)_2 \cdot 4H_2O$ (0.0046 *M*), and $MgSO_4 \cdot 7H_2O$ (0.0023 *M*); the total osmotic pressure of the solution was one atmosphere (**12**), and its reaction was

pH 4.5. After incubation the three samples were blanched and frozen prior to analysis. Following analysis the net increase in carotene could be calculated, since the initial weight of all four samples and the carotene content of the control were known. In this way the actual synthesis or loss of carotene could be expressed on an absolute basis independent of any changes in size or weight of the samples during incubation.

The incubating chamber consisted of a glass-topped refrigerator-incubator $6 \times 3 \times 5$ feet, equipped with a removable center partition. With the center partition in place, a different temperature could be maintained in each half of the box; when removed, the entire chamber could be kept at one temperature. The temperature could be controlled to within $\pm 0.5°$ C. The temperature of the leaves was estimated by a leaf-wrapped thermometer exposed to the same light intensity as the sample. The top of the incubator consisted of two sheets of glass separated by an air gap. One of the sheets was 0.25-inch heat-absorbing plate and the other 0.25-inch heat-tempered plate. This top permitted about 70% of the visible light to enter and kept out approximately 70% of the radiations longer then 7600 A. Illumination was provided by a 3-kilowatt mercury-vapor lamp (G.E. type AH-9) suspended 18 inches above the glass top. The characteristics of the lamp have been described by WEITZ and AMICK (**20**).

The analytical method used for carotenoid determinations was that of ZSCHEILE and WHITMORE (**26**) as modified by BERNSTEIN and THOMPSON (**2**) with a further change which consisted of employing 60% acetone in Skellysolve B to elute the carotenols from the column. As long as the acetone concentration in the Skellysolve solution of the pigments remained below 10–15%, the chlorophylls were completely retained by the layer of calcium hydroxide (Mallinckrodt NF XII) and showed no tendency to migrate down the column, even upon subsequent development of the column with eluants containing higher concentrations of acetone. The carotenes were eluted with 10% acetone in Skellysolve and the carotenols with 60% acetone in Skellysolve. An increase in density, as measured at 440 mμ, was the criterion used for carotene synthesis. Since carotenol values were calculated from the standard curve for carotene, the figures are to be considered simply as arbitrary units. Though carotenols were always measured, the results are given only if the response of carotenol synthesis to the experimental conditions differed markedly from that of carotene synthesis.

Results

VALIDITY OF LEAF-CULTURE TECHNIQUE

The use of detached leaves is in many respects undesirable, since the information really wanted is that which pertains to the intact plant. Some of the experiments reported here would have been impossible, however, using intact plants. In addition, the difficulties of orienting attached leaves normal to the light source, and the loss in precision owing to the impossibility of obtaining the initial weight of attached leaves, made the detached-leaf-culture technique desirable.

That no serious injury occurred to the carotene-synthesizing mechanism owing to the leaf-culture method was shown by an experiment designed to measure the course of carotene synthesis with respect to time. The net increase in carotene in unit time did not fall off for at least the first 32 hours (fig. 1, table 1, experiment I). In all subsequent experiments incu-

bation was stopped at the end of 24 hours. Another experiment was performed in which the amount of carotene synthesized by leaves attached to the stem of the plant, with the cut end of the stem immersed in nutrient solution, was

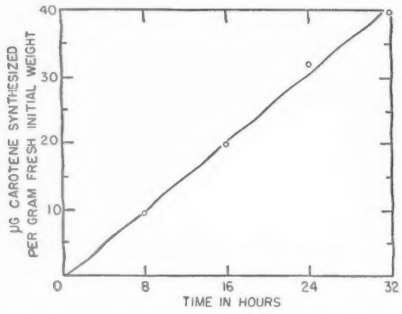

FIG. 1.—Carotene synthesis as function of time

compared with the amount synthesized by detached half-leaves. A parallel series was run in which the cotyledons were left attached to the stem. The presence or absence of the cotyledons made no significant difference in the amount of carotene synthesized by leaves attached to their stems (table 1, experiment II). Since the midribs of all leaves were removed, previous to weighing, the amount of carotene synthesized under both conditions should be directly comparable. The explanation for the much greater amount of carotene synthesized in the detached half-leaves than in the attached leaves probably lies in the fact that, when a leaf is severed from the stem, food reserves are not translocated from the leaves. The nutrient solution into which the cut ends of the stems were put was cloudy at the end of the 24-hour run and supported abundant bacterial growth, but the solution in which the half-leaves were floating was clear. It is known that detached leaves incubated in the dark lose very little carbohydrate compared with the leaves of intact plants treated similarly (**24**:33).

EFFECT OF LIGHT INTENSITY ON CAROTENE SYNTHESIS

In the following experiment an attempt was made to determine the effect of light intensity on the rate of carotene synthesis. Since the range of intensities obtainable by varying the shelf height in the incubator was small, reductions in light intensity were made by covering the

TABLE 1

COMPARISON OF CAROTENE SYNTHESIS IN LEAF CULTURES AND IN ATTACHED LEAVES*

Experiment no.	Treatment	Incubation time (hours)	No. of samples	Mean ± S.E.$_m$† of carotene synthesized/gm. fresh initial weight (μgm.)
I	Detached half-leaves	8	6	10.2 ± 0.91
		16	6	19.8 ± 1.87
		24	6	32.2 ± 4.28
		32	6	39.9 ± 4.22
II	Detached half-leaves	24	6	30.8 ± 0.60
	Attached whole leaves‡			
	+Cotyledons	24	5	6.8 ± 4.95
	−Cotyledons	24	5	9.8 ± 4.12

* Conditions of experiments: temperature, 30° C.; light intensity, 500–575 ft-c.

† Mean plus-or-minus standard error of mean.

‡ Initial weight of sample was assumed to be same as matched leaves used as controls for initial carotene content.

Petri dishes with a loosely fitting lid of one to several thicknesses of white paper, depending on the reduction in intensity desired. The results (table 2, experiment III, fig. 2) show that in darkness a small amount of carotene is lost, that the optimal light intensity lies between 400 and 600 foot-candles (measured with a Weston meter), and that there is a smaller net increase at 1000 and 1400 ft-c. The

TABLE 2

EFFECT OF LIGHT INTENSITY ON SYNTHESIS OF CAROTENE*

Experiment no.	Light intensity (ft-c.)	No. of samples	Mean ± S.E.$_m$ of carotene synthesized/gm. fresh initial weight (μgm.)
III. Young leaves	0	5	− 7.6 ± 1.16
	200	5	27.0 ± 1.14
	400	5	35.0 ± 2.11
	600	5	36.2 ± 3.19
	1000	4	16.0 ± 2.68
	1400	4	11.8 ± 2.68
IV. Older leaves	600	7	18.6 ± 0.81
	1000	7	18.7 ± 1.69
	1800	7	10.3 ± 2.34

* Conditions of experiment: temperature, 30° C.; incubation, 24 hours.

shape of the curve can be explained by an increase in the rate of a photo-destroying process relative to the rate of carotene synthesis (2), although a slower rate of synthesis at higher light intensities is not excluded.

The exact light intensity at which this carotene-destroying process becomes evident is influenced by the age of the leaf. When leaves approximately one-third expanded were used—instead of one-fifth to one-fourth, as in the other experiments—it was found that there was no lowering of the net increase of carotene with an increase in light intensity from 600 to 1000 ft.-c. (table 2, experiment IV). There was, however, a significant decrease in leaves exposed to 1800 ft-c. as compared with those exposed to 1000 ft-c. There was a slower rate of synthesis in older leaves, as compared with younger leaves, even with optimal light.

Carotenols behaved similarly to carotene in the experiment involving younger leaves, but in the somewhat older leaves there was an increase in carotenols only at 1800 ft-c. The data, involving the same number of samples as in experiment IV, were as follows: 600 ft-c., −2.6 ± 4.70; 1000 ft-c., −3.3 ± 3.92; and 1800 ft-c., 19.0 ± 3.60 units of carotenol synthesized per gram fresh initial weight.

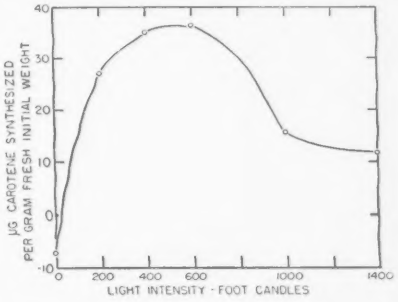

FIG. 2.—Carotene synthesis as function of light intensity.

It is evident that the net increase of carotene represents the difference between the amount synthesized and the amount destroyed during the incubation period. Since there is at present no way to measure independently the net carotene loss, the net increase is referred to simply as μgm. carotene synthesized.

In all subsequent experiments, unless otherwise indicated, the light intensity employed was 600 ft-c.

TEMPERATURE COEFFICIENT OF SYSTEM

In attempting to determine the temperature coefficient of carotene synthesis, one is faced with the same difficulties encountered in determining the tempera-

ture coefficient of photosynthesis, and, in addition, there are certain other complications. Thus, in the absence of definitive knowledge of the precursors of carotene no steps can be taken to guarantee that a temperature increase can produce its maximum effect. Table 3, experiment V, and figure 3, show the data obtained for two temperatures (approximately 25° and 35° C.) and five light intensities.

TABLE 3

TEMPERATURE COEFFICIENT OF CAROTENE SYNTHESIS AT SEVERAL LIGHT INTENSITIES

Experiment no.	Light intensity (ft-c.)	Temperature (° C.)	No. of samples	Means ± S.E.$_m$ of carotene synthesized/gm. fresh initial weight (μgm.)
V . . .	100	33	9	11.9 ± 1.38
		24	9	8.3 ± 1.82
	300	34	5	20.8 ± 1.07
		25	4	11.5 ± 2.22
	600	35	5	27.0 ± 1.67
		26	5	19.8 ± 1.24
	800	34	5	17.0 ± 3.20
		25	3	15.7 ± 0.35
	1200	36	4	4.0 ± 1.78
		29	5	10.2 ± 0.86

At 600 ft-c. an estimate of 1.4 is obtained for the Q_{10}, corrected for a 10° C. difference by means of the relationship,

$$\log Q_{10} = \frac{10}{t_2 - t_1} \log \frac{k_2}{k_1}.$$

This correction is not strictly accurate, but the factor, and thus the error, is small. At 1200 ft-c., where there is only a small net increase in carotene, the amount of carotene lost owing to a temperature-sensitive dark destruction becomes appreciable (table 8, experiment XV), thus causing the divergence of the two curves.

EFFECT OF EXTERNALLY SUPPLIED ORGANIC NUTRIENTS

The low rate of carotene synthesis found in leaves free to translocate material out of the leaf blade suggested that it might be possible to furnish organic nutrients in the culture solution which would be available for carotene synthesis. Since the time and temperature of incubation would permit the abundant growth of microörganisms, a bacteriostatic compound was added to the nutrient solution. WENT and CARTER (21) found that 0.025% sulfanilamide could

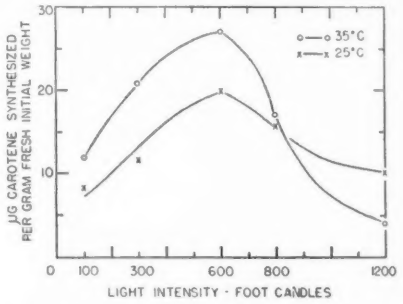

FIG. 3.—Carotene synthesis at two temperatures and five light intensities.

be used to suppress bacterial growth in sucrose solutions sprayed on plant leaves. An experiment was designed to determine if that concentration of sulfanilamide would be inhibitory to carotene synthesis. Table 4 shows the amount of carotene synthesized by leaves in the basic mineral nutrient and that synthesized by leaves in the basic mineral nutrient plus 0.025% sulfanilamide. The difference between the means of the two treatments is not significant. A concentration of 0.025% sulfanilamide was used in all the remaining experiments, therefore, regardless of whether organic nutrients were also added.

In the cultures without added organic nutrient an attempt was made to reduce

the amount of carotene synthesized to as low a level as possible so that the detection of the contribution of the nutrient might be more easily made. This was accomplished by reducing the CO_2 content of the air available to the cultures. A galvanized metal box (120-liter capacity) was provided with an outlet and inlet and a glassed-in cover which fitted into the oil seal of the box. The air was recirculated by a bellows pump which forced the air through a soda-lime tower. The condition of the soda-lime was determined by a very thin layer of caroxite,

TABLE 4

EFFECT OF SULFANILAMIDE ON CAROTENE SYNTHESIS

Experiment no.	Treatment	No. of samples	Mean ± S.E.$_m$ of carotene synthesized/gm. fresh initial weight (μgm.)
VI....	+Sulfanilamide	6	26.3 ± 2.09
	−Sulfanilamide	6	27.8 ± 1.28

an indicating CO_2-absorbent. In addition, several Petri dishes full of 30% sodium hydroxide were placed in the box. The effectiveness of this arrangement in reducing photosynthesis, as measured by the dry-weight method, depended on the length of time allowed between sealing the box and beginning illumination of the leaves.

Several experiments were performed to determine the effect of added glucose and sucrose on the amount of carotene synthesized in CO_2-poor air. The sugars were added to the basic mineral nutrient at a concentration of 0.15 *M*. The concentration of sulfanilamide was 0.025% in all the solutions. The results of these experiments are shown in table 5, experiments VII and VIII. Comparisons may be made only of the treatments within a single experiment, since the residual CO_2 of the incubator fluctuated from experiment to experiment and because the weather prevailing during the period of growth of the plants varied a great deal, thus influencing their condition at the beginning of the experiment.

Both sucrose and glucose were able to increase greatly the amount of carotene synthesized when supplied to leaves in

TABLE 5

EFFECT OF EXTERNALLY SUPPLIED ORGANIC NUTRIENTS ON CAROTENE SYNTHESIS

Experiment no.	Treatment	No. of samples	Mean ± S.E.$_m$ of carotene synthesized/gm. fresh initial weight (μgm.)
VII...	$-CO_2$ −sucrose	5	12.6 ± 1.08
	$-CO_2$ +sucrose	3	26.3 ± 1.67
	$+CO_2$ −sucrose	5	21.8 ± 1.11
	$+CO_2$ +sucrose	5	18.8 ± 2.35
VIII..	$-CO_2$ −glucose	5	14.8 ± 2.08
	$-CO_2$ +glucose	5	33.6 ± 1.43
IX...	$-CO_2$ −glycerol	5	23.4 ± 2.92
	$-CO_2$ +glycerol	5	10.2 ± 2.63
	$+CO_2$ −glycerol	2	43.0
X....	$-CO_2$ −pyruvate	6	23.0 ± 0.68
	$-CO_2$ +pyruvate	4	−12.8 ± 2.68
	$+CO_2$ −pyruvate	4	44.8 ± 2.17
	$+CO_2$ +pyruvate	1	− 2.0

CO_2-poor air. There are several possible interpretations of these data, but such discussion is postponed to the section on the use of poisons to inhibit carotene synthesis and to the "discussion" proper.

Experiments were also performed in which glycerol and pyruvate were supplied to the leaves. Both compounds were used at a concentration of 0.15 *M*, with all other conditions as previously described. The pyruvate was added to the nutrient solution as sodium pyruvate, prepared according to the method of ROBERTSON (14), except that the pyruvic acid used (E.K. Co. no. 498) was redistilled *in vacuo* (14 mm. Hg) previous

to neutralization. Only that fraction boiling between 160° and 165° C. (760 mm. Hg) was used. Under the conditions of these experiments both glycerol and pyruvate caused a decrease in the net amount of carotene synthesized as compared with the controls (table 5, experiments IX and X).

FROMAGEOT (**6**) studied the effect of glycerol on photosynthesis and respiration of the alga *Ulva lactuca*. He observed a depressing effect of glycerol on oxygen evolution in light at all concentrations up to 40%; at concentrations of over 40% there was an actual oxygen uptake. By interpolation from his graphs the concentration of glycerol used in the present experiment would cause an inhibition of oxygen evolution of about 12%. One might then interpret the present data as indicating that glycerol is not a suitable material for carotene synthesis—although this is not consistent with the data of INGRAHAM and STEENBOCK, working with *Mycobacterium phlei* (**8**)—and that the depressing effect of glycerol on photosynthesis caused a reduced carotene synthesis. The inhibition is more likely due to an effect of the relatively high glycerol content on the carotene-synthesizing mechanism. In this case the data for carotenol synthesis are not very helpful owing to the large variation in the results. The mean for the $-CO_2$ + glycerol cultures is 6.6 ± 5.38 and for the $-CO_2$ − glycerol cultures, 4.0 ± 6.19 units of carotenol synthesized per gram fresh initial weight.

In the experiment involving pyruvate the results cannot be easily interpreted. In this case there was an actual loss of carotene in the +pyruvate cultures. The leaves were apparently not injured, as evidenced by their normal appearance and by the fact that the +pyruvate leaves gained 74% in fresh weight as compared with a 68% increase for the −pyruvate cultures. Furthermore, the synthesis of carotenols in the +pyruvate cultures amounted to 16.5 ± 4.25 compared with 4.0 ± 2.55 units for the −pyruvate cultures. The observation that neither glycerol nor pyruvate can contribute to carotene synthesis under the conditions employed cannot be considered proof that they lie outside the synthetic pathway until more data are available, particularly as to the effect of different concentrations of these compounds.

DIFFERENTIAL EFFECTS OF POISONS AND OF LOW CARBON DIOXIDE CONCENTRATIONS ON PHOTOSYNTHESIS AND CAROTENE SYNTHESIS

In the preceding section it has been shown that glucose and sucrose can increase carotene synthesis in CO_2-poor air. These experiments do not enable one to choose between the possibilities of the carbohydrate entering directly into the synthetic mechanism or of its being first oxidized, or photoxidized, to CO_2 and then entering into the normal photosynthetic mechanism. Therefore, certain experiments were performed in which the treatments might serve to act differentially on carotene synthesis and photosynthesis. The first such experiment consisted of a study of the effects of hydroxylamine on carotene synthesis in the presence and absence of sucrose. Photosynthesis is extraordinarily sensitive to hydroxylamine, being almost completely inhibited by concentrations as low as 0.001 *M* (**17**). Respiration is usually not affected by such low concentrations. The concentration of hydroxylamine used was 0.001 *M* (added to the nutrient as the hydrochloride), and of sucrose 0.15 *M*, with all other factors as previously described. The same experiment was per-

formed using glucose as the carbohydrate source. These results, however, are suspect, owing to the almost certain formation of glucose oxime.

A measure of the inhibition of carotene synthesis subsequent to photosynthesis can be obtained by comparing the + and −hydroxylamine cultures in the absence of sucrose (table 6, experiment XII). This inhibition amounted to 26 μgm., or 74%. The difference between

TABLE 6

EFFECTS OF HYDROXYLAMINE AND FLUORIDE ON CAROTENE SYNTHESIS

Experiment no.	Treatment	No. of samples	Mean ± S.E.$_m$ of carotene synthesized / gm. fresh initial weight (μgm.)
XI	$-NH_2OH$ −glucose	5	26.8 ± 1.71
	$-NH_2OH$ +glucose	5	37.4 ± 2.37
	$+NH_2OH$ −glucose	4	2.8 ± 1.11
	$+NH_2OH$ +glucose	6	36.5 ± 2.61
XII	$-NH_2OH$ −sucrose	5	35.4 ± 2.91
	$-NH_2OH$ +sucrose	5	49.6 ± 1.96
	$+NH_2OH$ −sucrose	5	9.0 ± 1.51
	$+NH_2OH$ +sucrose	6	31.8 ± 2.04
XIII	−NaF −glucose	8	34.2 ± 2.24
	−NaF +glucose	4	48.2 ± 2.18
	+NaF −glucose	4	23.5 ± 2.98
	+NaF +glucose	7	21.6 ± 3.92

the + and −sucrose cultures in the presence of hydroxylamine was 23 μgm., or an increase of 72% due to the sucrose. That the hydroxylamine was still active is shown by the difference between the + and −sucrose treatments. For the interpretation to be completely valid it would be necessary to measure manometrically the actual depression of photosynthesis by hydroxylamine, since there is the possibility that the breakdown products of the sucrose themselves reacted with the hydroxylamine. It has been shown (10), however, that when glucose is fed to a leaf, it is converted in large measure to sucrose, so that it is unlikely that there would be a large-scale conversion of sucrose into reducing sugars which could then form oximes. In addition, tests for the presence of reducing substances in the nutrient following incubation were almost totally negative.

If the assumption is made that the degree of inhibition of photosynthesis by hydroxylamine is the same in the presence and absence of sucrose, then it appears that the contribution of sucrose to carotene synthesis is not impaired by a concentration of hydroxylamine which inhibits photosynthesis.

TABLE 7

PERCENTAGE REDUCTION OF PHOTOSYNTHESIS AND CAROTENE SYNTHESIS IN CO_2-POOR AIR

Experiment no.	Carotene synthesis	Photosynthesis
VII	42	80
IX	46	97
X	49	49

In addition, it was found in the experiments in which leaves were incubated in CO_2-poor air that the reduction in photosynthesis as measured by the dry-weight method was not always proportional to the reduction in carotene synthesis. Table 7 shows these data.

It can be seen that in two of the three cases the reduction of photosynthesis was far in excess of the reduction in carotene synthesis, amounting in one case to a 97% reduction in photosynthesis while 54% of carotene synthesis remained intact. The variation in the proportion between the two columns could be explained on the basis of the varying amount of carbohydrate with which the leaves entered the experiment.

When negative results are obtained with respect to the sensitivity of a system to a poison, they are often not reported because of the difficulty of estab-

lishing that the poison entered the cell. It seems, however, to be agreed among students of photosynthesis that the system is not sensitive to small amounts of fluoride. It was, therefore, of interest to determine whether carotene synthesis was sensitive to fluoride. The experimental conditions were exactly as reported in the preceding experiments except that it was necessary to omit the $MgSO_4$ and $Ca(NO_3)_2$ from the nutrient solution to avoid precipitation of the fluoride. The concentration of sodium fluoride was 0.001 *M*. The results are reported in table 6, experiment 13. It can be seen by comparing the values obtained for carotene synthesis in the −glucose −fluoride cultures with those obtained in the −glucose + fluoride cultures that an inhibition of 29% could be attributed to the fluoride. If one compares the amount of carotene synthesized in the cultures furnished glucose in the absence of fluoride with the control, it can be seen that glucose made a contribution to carotene synthesis. A comparison of the −glucose + fluoride cultures with the +glucose + fluoride cultures shows that this contribution was completely halted by fluoride. This can be explained in at least three ways: (*a*) the compound available for carotene synthesis as the result of photosynthesis is not glucose, (*b*) fluoride prevents the entrance of glucose into the plant cell, or (*c*) the inhibition by fluoride is of a complete'y noncompetitive type which permits only so much carbohydrate to pass the block regardless of the concentration of the carbohydrate. The present data are insufficient to enable one to choose among these explanations.

Evidence for dark synthesis of carotene

It has been well established that etiolated leaves contain some carotene (**5**) and that there is a slow increase of this pigment in the dark. Therefore, a dark synthesis does exist under the conditions which prevail in an etiolated plant. It was desired, however, to ascertain if carotene could be made in the dark by green leaves, from externally supplied glucose. Strain (**18**), using etiolated barley seedlings supplied with sucrose, found that no synthesis could be observed. Since the amount of dark synthesis from added glucose observed in the present work was very small, it is easy to understand why conflicting reports might be obtained. Incubation was for 24 hours in absolute darkness at 30° C. During the preparation of the samples they received about one-half hour of very subdued illumination. The results are presented in table 8, experiment XIV, and figure 4.

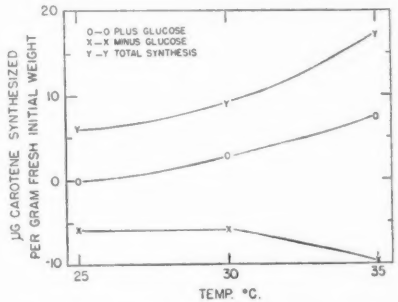

Fig. 4.—Carotene synthesis in darkness in presence and absence of glucose at 25°, 30°, and 35° C.

It is clearly evident that there is a difference between the + and −glucose cultures, the odds in this case attaining a value of 3.1×10^{15}:1 against so great a difference being due to chance. The real question, though, is whether the mean of the +glucose cultures is different from zero. The 1% fiducial limits, for the 30° C. experiment, are 0.6 to 5.0 μgm. carotene, so the mean is different from zero at odds of 99:1. There remains the

possibility that a slight loss of carotene occurs in the 2-minute blanching period and that glucose acts to prevent this destruction, thus giving an apparent increase. This is considered unlikely.

Additional evidence for a dark synthesis of carotene was obtained in a field experiment involving leaves left attached to the plant. The plants were grown during the early fall of 1947. The experiment was repeated twice using half-expanded lateral leaflets, in the one case the first trifoliate leaf, and, in the other, the second trifoliate leaf of a similar physiological age. A sample consisted of ten of the lateral leaflets of the trifoliate leaf picked from ten different plants. The remaining ten leaflets were left attached to the plant and were then harvested at a later time. The difference in carotene content is expressed on a per-sample basis, so that growth changes would not obscure the results (table 9).

TABLE 8

SYNTHESIS OF CAROTENE IN DARK FROM ADDED GLUCOSE

Experiment no.	Treatment	Temperature (° C.)	No. of samples	Mean ± S.E.$_m$ of carotene synthesized/gm. (fresh initial weight (μgm.)
XIV	−glucose	30	16	−6.25 ± 0.85
	+glucose	30	17	2.80 ± 0.75
XV	−glucose	35	5	−9.80 ± 2.59
	+glucose	35	5	7.40 ± 1.08
	−glucose	25	5	−6.20 ± 1.53
	+glucose	25	6	−0.20 ± 1.47

TEMPERATURE COEFFICIENT OF DARK SYNTHESIS

An attempt was made to determine the temperature coefficient of the dark synthesis. Detached half-leaves were used again with other conditions as previously described. Two temperatures were used, 25° and 35° C. In +glucose cultures there was a net increase in carotene at 35° C. and no net change at 25° C. (table 8, experiment XV, and fig. 4). In −glucose cultures there was a net decrease of carotene at both temperatures, although less at the lower temperature. It is thus apparent that both the processes of carotene synthesis and of carotene destruction are temperature-sensitive and that the Q_{10} is different for each reaction. Since it is not known whether any carotene is synthesized in the absence of glucose, or whether the rate of carotene destruction is the same in the presence and absence of glucose, it is difficult to calculate the temperature coefficient. If the assumption is made that the rate of loss of carotene is the same in the presence or absence of glucose, the true amount of carotene synthesized is the absolute difference between that gained with glucose and that lost in the absence of glucose. This is shown in figure 4 as "total synthesis." Through a comparison of the totals for 35° C. and 25° C., one obtains a temperature coefficient of 2.9. If it is further assumed that no carotene was synthesized in the −glucose cultures, then the Q_{10} for

TABLE 9

SYNTHESIS OF CAROTENE IN ATTACHED LEAVES

Experiment no.	No. of pairs	Time of harvest	Mean difference ± S.E.$_d$ of matched pairs harvested at indicated time (μgm.)
XVI	12	8:00 P M	
		6:00 A M	+ 9.2 ± 3.10
	11	6:00 A.M.	
		10:00 A.M.	+33.7 ± 3.00
XVII	12	8:00 P.M.	
		6:00 A.M.	+ 8.8 ± 3.75
	11	6:00 A.M.	
		10:00 A.M.	+12.3 ± 2.62

the dark destruction is 1.6 for the range 25°–35° C.

Discussion

It has been shown that the process of carotene synthesis in leaves can be advantageously studied for periods of at least 24 hours by using detached half-leaves floating on a suitable nutrient. In addition to the ease with which the leaves can be oriented normal to the light, and evenly illuminated, there is the advantage of the higher rate of carotene synthesis in detached leaves as compared with leaves attached to the plant stem. The extent to which data obtained from leaf cultures can be extended to the intact plant is unknown, but it is considered probable that such differences as do exist are differences of degree rather than kind.

Before proceeding to a discussion of the results obtained it would be well to re-emphasize that the net increase or decrease of carotene observed under the experimental conditions probably represents the sum of the carotene synthesized minus the carotene destroyed or in some way rendered unextractable. Two ways in which carotene destruction may occur in living leaves have been established. There is, first, the destruction of carotene which occurs in the dark. Since at higher temperatures in the presence of glucose a net increase of carotene has been observed, it appears that this destruction is being counteracted by a simultaneous synthetic process. Second, there is evidence for a photodestruction as shown by the smaller net increase in young leaves exposed to light intensities higher than 600 ft-c. In older leaves which at the start of the experiment would have a larger store of oxidizable cellular components, as well as a thicker cuticle, the intensity at which photodestruction first becomes evident is increased to something over 1000 ft-c. The third possible way in which carotene destruction might occur is by participation in some metabolic reaction in which the pigment is used up.

It must also be mentioned that photautoxidation of chlorophyll is known to be prevented by an external supply of oxidizable substrate. Thus, the addition of hydroxylamine or the depletion of carbon dioxide might act to decrease the amount of cellular materials and thus permit destruction of carotene. Similarly, the addition of glucose or sucrose would act simply to protect the pigment from photautoxidation, thus giving the appearance of a greater rate of synthesis. The observation that the addition of glycerol or pyruvate can decrease the rate of accumulation of carotene cannot be explained on the above basis, unless it is assumed that there was an inhibition of photosynthesis which was relatively greater than the inhibition of photautoxidation. Photautoxidation cannot be ruled out completely as an explanation of the observed phenomenon unless it were possible to measure independently carotene destruction and carotene synthesis. Neither does it appear, on the basis of existing knowledge and the present data, to be the most logical explanation of the effects of the various treatments on carotene synthesis. First, true photautoxidation is a phenomenon which is usually associated with much higher light intensities than were employed in the present work. MEYERS and BURR (11) studied photautoxidation in *Chlorella* over a wide range of intensities. Even in dilute suspensions of this alga no evidence for a depression of photosynthesis was ever obtained until the light intensity employed reached the point of light satura-

tion, that is, 1000–4000 ft-c. Second, the curve showing carotene synthesis as a function of time is a straight line for at least 32 hours. If photautoxidation of the carotene occurred at the intensity of illumination employed, 600 ft-c., it would have to consume a constant proportion of the carotene being synthesized despite the fact that leaf sugars and other oxidizable cellular compounds must have been increasing. Third, the experiment with fluoride showed a complete inhibition of the contribution of glucose to carotene synthesis. A higher net increase in carotene should have been observed if the only contribution of glucose was to protect the carotene from photautoxidation.

The interpretation of the contribution of externally supplied carbohydrates to carotene synthesis presents a problem which is analogous to that encountered in the utilization of organic acids by succulents (**13**). There it has been found that organic acids accumulate during the night and then gradually disappear during the day with the simultaneous formation of carbohydrates. This has been interpreted to mean either that the organic acids are photochemically reduced to carbohydrates or that they are oxidized (or photoxidized) to CO_2 and then enter directly into the usual photosynthetic path. In the present work the same alternatives apply. They are represented by equations (1) and (2).

Equation (1) would signify that in the normal leaf carotene is synthesized directly from some photosynthetic product, before the stage of stability represented by a carbohydrate is reached. Equation (2) indicates that carotene synthesis is a light-accelerated reaction, separable from photosynthesis and involving compounds accumulated in previous photosynthetic activity. The symbol $P_{1 \ldots n}$ represents a series of unknown intermediates.

Equation (2) has been adopted as a working hypothesis for the following reasons: (*a*) It was found in experiments in which leaves were incubated in CO_2-poor air that the reduction in photosynthesis, as measured by the dry-weight method, was not always proportional to the reduction in carotene synthesis. In one case a reduction of photosynthesis of 97% occasioned a reduction in carotene synthesized of only 47%. This indicates that a precursor of carotene must be stored in the leaf. (*b*) In leaves left attached to the stem and thus free to translocate carbohydrates out of the leaf blade, the rate of carotene synthesis was lower than in detached leaves in which carbohydrates must accumulate. (*c*) That a dark synthesis is possible indicates that carotene synthesis is separable from photosynthesis. Since this dark synthesis took place only when glucose was supplied, it would indicate that a sugar must play the same role in normal unstarved leaves. (*d*) In the experiment in which the leaves were poisoned with hydroxylamine in the presence and absence of sucrose, it was found that the synthesis of

$$\text{sugar} \xrightarrow[\text{photoxidation}]{\text{respiration}} CO_2 \xrightarrow[\text{(light)}]{\text{photosynthesis}} P_{1 \ldots n} \longrightarrow \text{carotene} \qquad (1)$$

$$CO_2 \text{ (atmosphere)} \uparrow CO_2$$

$$CO_2 \xrightarrow[\text{(light)}]{\text{photosynthesis}} \text{sugar} \longrightarrow P_{1 \ldots n} \xrightarrow[\text{(light accelerated)}]{} \text{carotene} \qquad (2)$$

$$\text{sugar} \text{ (externally supplied)} \uparrow \text{sugar}$$

the dark destruction is 1.6 for the range 25°–35° C.

Discussion

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$$\uparrow \text{sugar (externally supplied)}$$

carotene from externally supplied sucrose was not inhibited to the same extent as was the synthesis of carotene subsequent to photosynthesis. This experiment is not unambiguous owing to the possibility of a reaction between hydroxylamine and any reducing substance formed by the leaves from sucrose. (*e*) The sensitivity of the synthetic mechanism to 0.001 *M* sodium fluoride suggests a relationship between carotene synthesis and carbohydrate metabolism rather than between carotene synthesis and photosynthesis.

An interpretation such as this is of interest for two reasons: (*a*) The prospect of obtaining more information on the mechanism of synthesis of carotene is considerably enhanced if there is at least one step in the synthetic mechanism in which stable compounds such as carbohydrates are involved, and (*b*), since the reaction is light-accelerated, it would appear to be another example of a photochemical reduction occurring in leaves in addition to the known photochemical reduction of carbon dioxide.

Summary

1. A method is described for the study of the synthesis of carotenoid pigment in detached green leaves; it is applicable to experiments as long as 24 hours and is sufficiently precise to detect small increases or decreases of pigment on a per-sample basis.

2. The optimum light intensity for carotene synthesis under these conditions is 600 foot-candles.

3. The temperature coefficient of carotene synthesis at 600 foot-candles over the temperature range of 25°–35° C. is 1.4.

4. The dark synthesis of carotene in the presence of glucose occurs only one-fifteenth as rapidly as the corresponding synthesis in light.

5. There is a slow destruction of carotene in the dark in detached leaves.

6. The estimate of the temperature coefficient of the dark synthesis is 2.9 and of the dark destruction 1.6.

7. Externally supplied glucose and sucrose can contribute to carotene synthesis probably without first being oxidized to carbon dioxide.

8. There is at least one step in the synthesis of carotene which is fluoride-sensitive.

9. It is postulated that carotene synthesis may be represented by the equation shown below, in which $P_{1 \ldots n}$ represents an unknown series of intermediates.

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$$CO_2 \xrightarrow[\text{(light)}]{\text{photosynthesis}} \text{sugar} \longrightarrow P_{1 \ldots n} \xrightarrow[\text{(light accelerated)}]{} \text{carotene}$$

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METABOLISM OF RADIOACTIVE ELEMENTAL SULFUR APPLIED TO LEMONS AS AN INSECTICIDE

F. M. TURRELL AND MARCELLA CHERVENAK

Introduction

Elemental sulfur was applied to plants to control pests as early as 1824 (**58**), and it since has been generally recognized that sulfur dusts or sprays will cause injury ("sulfur burn") to plants during very hot weather. How this injury occurs has received little attention, though efforts to "safen sulfur" have been numerous as have efforts to explain its toxicity to fungi and insects.

Data accumulated over the past several years in this laboratory on the reaction of citrus fruit with elemental S dust show certain striking similarities of this reaction to the metabolism of S in certain higher and lower animals and plants, as well as to the reaction of higher plants with S gases.

REVIEW OF LITERATURE.—Human beings fed elemental S (colloidal) in the diet excreted almost quantitatively the

amount fed, as SO_4 in the urine (25). Ten per cent of elemental ground S taken orally by man is excreted in urine as inorganic SO_4 and in organic combination (11). Dogs fed elemental S in food excreted larger quantities of SO_4 in the urine than those not so fed (16), rats fed radioactive colloidal S excreted radioactive SO_4 in the urine (66), the bacterium *Thiobacillus thiooxidans* transforms elemental S to SO_4 (83, 84), and citrus fruit dusted with elemental S produces SO_4 (75).

When a salve of elemental S is applied to injured human skin, blood and urine show the presence of H_2S (8). Rabbit, cat, dog, cow, and pig liver form H_2S when combined with elemental S (59), as do whites of eggs (*Hühnereiweiss*) (57). We have found that earthworms and sow-bugs ground with elemental S produce H_2S gas, as might be expected, since certain insects contain glutathione (22). The leaves of a large number of such higher plants as apple, gooseberry, rose, thistle, etc. (7), such lower plants as the algae, *Pleurococcus* and *Ulothrix* (7), bryophytes, as *Polytrichum* and *Marchantia* (7), such fungi as *Penicillum*, *Saccharomyces*, *Sclerotinia*, etc. (7), such bacteria as *Thiobacillus oxidans* and *T. thioparus* (64), spores of such fungi as *Uromyces*, *Macrosporium*, etc. (41), and the sporophores of three species of Agaricaceae (41) produce H_2S gas from elemental S. Citrus fruit dusted with elemental S produces H_2S (72).

So far as the writer knows, no literature is available on the production of SO_2 gas by higher or lower animal or plant tissue on treatment with elemental S. Citrus fruit dusted with elemental S, however, produces small amounts of SO_2 gas (80).

Many papers have appeared which show that elemental S is toxic to man (36), to such higher animals as white rats (37), to such lower animals as tent caterpillars (46), to southern and fall army worms and cutworms (17), during hot weather to such higher plants as citrus (45), pears (60), grapes (10), apple microspores (pollen) (44), sugar-beet microspores (4), and to such lower plants as fungi (24, 28) and to their conidia, urediospores, and spores (38, 43, 88), and to citrus fruit (76). Its action is generally thought to be an indirect effect.

H_2S gas is toxic to man (30, 85), to such higher animals as rats and mice (86), such lower animals as house flies (86), beetles (52), protozoa, rotifers, and crustacea (50), *Passalus* (1), such higher plants as tomato, buckwheat, and tobacco (71), such lower plants as ascomycetes, fungi imperfecti, yeast, and bacteria (2, 18, 19, 42), such parts of lower plants as sclerotia (42), and to citrus fruits (74).

SO_2 gas is toxic to man (30), to such higher animals as rats and mice (86), such lower animals as house flies (86), *Passalus* (1), such higher plants as alfalfa, orchids, and pine trees (32, 48, 89), such lower plants as fungi, yeasts, and bacteria (42), and to citrus fruit (78).

Sulfides injected as H_2S have been shown to be oxidized in dogs (27), and Na_2S injected in dogs increased the SO_4 in the blood (15), but no literature seems to be readily available on the products formed in lower animals. H_2S gas is oxidized to SO_4 with an accompanying lowering of the pH value in tissue in citrus fruit (78, 80); this could probably occur generally in the tissues of higher plants, particularly if the buffer capacity of the tissue is low. In such bacteria as *Beggiatoa* H_2S is oxidized to SO_4 (34); also in the purple sulfur bacteria (82).

No literature has been found on the conversion of SO_2 gas to SO_4 in man, higher animals, or lower animals, al-

though liver, kidney, and chorion slices of the rat have been shown to convert thiosulfates to SO_4 (**56**). SO_2 gas is converted to SO_4 by such higher plants as alfalfa (**67**); a lowering of the pH value of the expressed plant fluid was observed after SO_2 injury in alfalfa and dahlia leaves (**70**). Radioactive SO_2 absorbed by wheat, barley, and corn leaves is oxidized to S^*O_4, synthesized into organic S* compounds, transformed to S^*O_4, translocated as such, and transformed back into organic S* compounds in roots and grain (**69**). Although no references have been found on the conversion of SO_2 to SO_4 by lower plants, certain fungi as *Aspergillus* and *Botrytis* convert thiosulfates to SO_4 with a release of H_2S and a lowering of the pH values of the media (**3**). *Thiobacillus thiooxidans*, when grown on mineral media containing thiosulfate, makes the medium more acid (**63**), and *T. thioparus* under similar conditions produces SO_4 (**63**). Citrus fruits oxidize SO_2 to SO_4 (**77, 80**).

Higher animals, such as dogs, injected with Na_2SO_4, showed very little absorption of the SO_4 ion in blood, muscle, or viscera (**14**), but in dogs and rabbits, when $MgSO_4$ and Na_2SO_4 were injected or fed via intestine, the kidney retained the SO_4 ion (**13**). An experiment in which Na_2SO_4, labeled with O^{18}, was injected into rabbits, indicated that most of the SO_4 was excreted in the urine and none or only a small amount exchanged in the tissue (**5**).

According to LOEB (**40**), lower animals as a rule require SO_4 in their nutrition. He found that addition of $MgSO_4$ to artificial culture media greatly increased the survival of *Drosophila* reared on them. SO_4 is readily absorbed from sea water by the ascidian, *Chelyosoma*, which concentrates it to as high as 0.88 normal H_2SO_4 (**35**). Other than that SO_4 occurs in the urine of certain insects as an end product of sulfur metabolism (**87**), little information appears to be available on the intake and utilization of SO_4 in lower animals. Higher plants absorb SO_4 from the soil through the roots and convert this to sulfites and —SH compounds, such as glutathione, cysteine, etc. (**49**). Radioactive S^*O_4 was shown to be absorbed by wheat, barley, and corn from nutrient solution, translocated to the leaves, changed to cystine S*, insoluble organic S* and acid-soluble (noncystine) organic S*, then transformed back into S^*O_4, retranslocated to roots or grain, and resynthesized to organic S* (**69**). Lower plants, such as yeasts, obtain S as SO_4 most readily from nutrient media and synthesize protein (**65**). *Microspirum* (**81**) and *Sporovibrio* (**12**) reduce SO_4 to H_2S.

From this review it appears that the metabolism of elemental S applied as dust or of S applied as H_2S or SO_2 gas to citrus fruits shows certain similarities to the metabolism of S in a wide variety of living organisms. There are, too, many dissimilarities in the sulfur metabolism within the categories of organisms mentioned, to which we purposefully have not called attention.

GUTHRIE (**26**) showed that glutathione reacts quantitatively with elemental S to produce H_2S gas. It was shown by TARVER and SCHMIDT (**66**), using radioactive elemental S, that $2\ RSH + S^* \rightarrow RSSR + H_2S^*$. Using citrus fruit we have shown previously (**80**) that

$$S + \text{fruit} \longrightarrow H_2S + SO_2 + SO_4$$
$$H_2S + \text{fruit} \longrightarrow SO_2 + SO_4$$
$$SO_2 + \text{fruit} \longrightarrow SO_4$$

In order to trace the source of the S involved in these reactions, we have used radioactive S^{35} in the following experiments.

Material and methods

Radioactive sulfur (S^{35}) with a half-life of 87.1 days, emitting beta rays with upper energy limits of between 0.12 and 0.17 M.E.V., was obtained from the Atomic Energy Commission as Na_2S^*. The elemental S* obtained therefrom was dissolved in 150 ml. of CS_2. No exchange between CS_2 and S* occurs by such treatment (9). This solution was then poured over 30 gm. of 325-mesh elemental ground S, the CS_2 evaporated at room temperature, and the elemental S* reground in a glass mortar. This was applied to two sets of twenty-eight freshly picked, ripe, yellow Eureka lemons which were used as described in each of the following experiments.

The procedures used in treating the fruit and fruit products in both experiments I and II were as follows: The evolved H_2S^* was absorbed in a 2% zinc acetate solution, was converted to S^*O_4 with an alkaline solution of 30% H_2O_2, and precipitated as BaS^*O_4. The evolved S^*O_2 was absorbed in a phosphate-borate buffer solution (68), was similarly oxidized, and precipitated with $BaCl_2$ as BaS^*O_4.

S* dust was washed from the intact lemons with a soft brush and three washings of distilled water. These were followed by a quick dip in 95% ethanol, after which the fruits were wiped dry with a soft clean towel. The S* from these washings was combined, crystallized from CS_2, and weighed. S^*O_4 determinations were made on the dried, ground peel by oxidizing with $Mg(NO_3)_2$, and precipitating with $BaCl_2$ in accordance with standard methods.

The specific activity of the elemental S* dust was determined on BaS^*O_4 after oxidation of the S* to S^*O_4 by refluxing in concentrated HNO_3 for several hours.

All samples were counted as BaS^*O_4 at a density as near the recommended optimum of 15 mg./cm.^{2} as possible (31). This value was too high and appears to be an error; consequently, all counts were corrected for self-absorption. Duplicate counts of 10,000 or more were made on each sample using a Cyclotron Specialties Company scaling unit, a Neher-Harper preamplifier, and a Cyclotron Specialties Company Geiger counter tube having a relatively thin mica window.

The counting setup was standardized using a radium D + E standard supplied by the United States Bureau of Standards and a sample of C^{14}-labeled $Ba(C_2H_3O_2)_2$ kindly supplied by Dr. HORACE A. BARKER of the University of California at Berkeley. By extrapolating the Ra D + E curve based on counts with varying thicknesses of aluminum foil to zero absorber, the tube geometry was determined. The geometry of the tube using the C^{14} sample was then found, and finally the geometry of the tube for S* was estimated by plotting the two geometries against the respective beta-ray energies of the standards. It was estimated that the tube geometry was 0.016. This value was then used in making up the balance sheet of the S* applied versus the S* counted.

EXPERIMENT I. FRUIT IN CONTACT WITH S*.—These fruits (twenty-eight lemons) were dusted a few at a time by shaking them in a bag containing the elemental S* dust. They were then placed in a large bottle in a thermostat at 106° F. The large bottle was fitted with an absorption train to absorb H_2S, SO_2, and NH_3 from the air pulled into the bottle containing the fruit. An additional absorption train was used to absorb the H_2S, SO_2, and NH_3 formed by the fruit. Movement of the gases was effected with a rotary vacuum pump, and the gases

were slowly drawn through the apparatus for the 68-hour incubation period. A full description of the apparatus and of the method will be found in the paper of TURRELL (**79**).

Histological sections of the peel were made by killing 0.25-cm.² blocks of the rind in a mixture consisting of 6.5 ml. 40% formalin, 2.5 ml. glacial acetic acid, and 100 ml. 50% ethanol for 24 hours, washing out the killing agent in water, dehydrating in a butyl alcohol series, imbedding in paraffin, and preparing microtome sections 15 μ in thickness.

Radioautographs were made of radial sections of the peel of fruit treated with radioactive sulfur by placing regular periapical dental X-ray film (safety base) in direct contact with the paraffin sections mounted on microscope slides. The film was held in place with an additional microscope slide, and tight contact maintained by binding the slides together with rubber bands. The slides were subsequently placed in a refrigerator for 111 days before development in an Eastman D-8 developer.

EXPERIMENT II. FRUIT NOT IN CONTACT WITH S*.—Approximately 10 gm. of elemental S^{35} dust in an uncovered Petri dish 9 cm. in diameter was placed in the bottom of a large bottle. The lemons were piled around the Petri dish in such a way as not to come in direct contact with elemental S*. Accidental direct contact was prevented by placing a simple arrangement of glass rods above the Petri dish. The bottle containing the fruit was placed in a thermostat at 106° F. as in experiment I and connected with the gas-absorption train.

After 43.5 hours' aspiration the sulfur collected as H_2S and SO_2 was converted to $BaSO_4$. No $BaSO_4$ precipitate was found for either H_2S or SO_2. It was thought that the small amount of S vapor formed may have been drawn off in the air stream during aspiration. Consequently, the reaction bottle was closed off from the absorption trains, and incubation continued for a period of 30 hours. At the end of this time the gases from the reaction bottle were allowed to escape through the train and finally were pumped through.

Results

EXPERIMENT I. FRUIT IN CONTACT WITH S*.—When lemons were dusted with elemental radioactive S and incubated, all the products formed as H_2S, SO_2, and SO_4 in the peel and as SO_4 in the exudate were found to be radioactive (table 1).

TABLE 1

SPECIFIC ACTIVITY OF PRODUCTS FORMED BY DUSTING EUREKA LEMONS WITH ELEMENTAL RADIOACTIVE SULFUR (S^{35}) AND INCUBATING AT 106° F. CORRECTED FOR BACKGROUND, DECAY, AND SELF-ABSORPTION

Product formed	Specific activity*	Per cent of specific activity of S^{35}	Specific activity/ cm^2†/hr $\times 10^{-7}$
S^{35}‡	0.1107		
H_2S^*	0.1136	102.5	7.18
S^*O_2	0.0159	14.4	1.01
S^*O_4 (peel)	0.0853	77.1	5.39
S^*O_4 (exudate)	0.0915	82.7	5.79

* Counts/sec/mg BaS^*O_4.
† Based on surface area of fruit ≡ area of S* dust.
‡ S* applied to fruit as dust.

Comparison of the specific activities of the H_2S^* and S* shows that all the S* in the H_2S^* was derived (within the error of measurement) from the S* applied (table 1). This suggests that the H_2S^* formed from citrus fruit may be due to the reaction

$$2RSH + S^* \longrightarrow RSSR + H_2S^*$$

demonstrated *in vitro* (**66**).

Comparison of the specific activities of the S^*O_2 and S^* shows that 14% of the S^*O_2 was derived from the S^* applied (table 1), and, therefore, most if not all of the S^*O_2 must have been split off from some compound in the fruit peel.

If the specific activities of the S^*O_4 in the peel and of the S^*O_4 in the exudate formed during incubation of the fruit are compared with the specific activity of the S^* applied, it can be seen that 77% and 83% of the peel- and exudate-SO_4 activity, respectively, were derived from the elemental S^* applied (table 1). The latter values suggest dilution of the radioactive S^*O_4 with the inactive SO_4 normally present in the peel.

TABLE 2

SPECIFIC ACTIVITY OF PRODUCTS FORMED BY EXPOSING EUREKA LEMONS TO VAPORS OF ELEMENTAL RADIOACTIVE SULFUR AT 106° F. CORRECTED FOR BACKGROUND, DECAY, AND SELF-ABSORPTION

Product formed	Specific activity*	Per cent of specific activity of S^{35}	Specific activity/ cm^2†/hr $\times 10^{-7}$
S^{35}‡	0.1107		
H_2S^*	0.00625	5.65	32.8
S^*O_2	0.00108	0.98	5.67
S^*O_4 (peel)	0.00204	1.85	10.7

* Counts/sec/mg BaS^*O_4.
† Surface area of S^* from which vapors formed.
‡ S^* from which vapor was formed.

EXPERIMENT II. FRUIT NOT IN CONTACT WITH S^*.—The specific activities of the products formed, such as H_2S^*, S^*O_2, and S^*O_4, when lemons were exposed to the vapors of radioactive elemental S^* contained in an open Petri dish are shown in table 2. As found in the preceding experiment, all the products were radioactive, that is, H_2S^*, S^*O_2, and the S^*O_4 from the peel. This suggests that elemental S vapor will penetrate lemon-fruit peel. The specific activities were considerably lower, however, for the H_2S^*, S^*O_2, and S^*O_4 formed from the S^* vapor (table 2) than in the former experiment when the S^* was in contact with the peel (table 1).

There are five possible explanations of the low specific activities in experiment II: (*a*) the rate of vaporization of S^{35} may have been lower than that of the S^{32}, owing to the molecular weight of the former being about 9.4% greater than the latter;[1] (*b*) preferential adsorption of S^{35} on the relatively large surface (5,670 $cm.^2$) of the Pyrex glass bottle containing the fruit may have been greater than that of the S^{32};[1] (*c*) the preferential action of thiol enzymes on S^{32} may have been greater than on S^{35}, the S^{35} having a lower zero point energy requiring greater activation energy;[1] (*d*) the S in H_2S, SO_2, and SO_4 may have been derived from inactive S^{32} components before the same products were formed from S^{35} applied; and (*e*) more of S_8^{35} may have remained near the bottom of the bottle than did the S_8^{32}.

Although the incubation temperatures were the same in experiments I and II, the fruit surfaces were 2322 $cm.^2$ in experiment I, where the fruit were S^*-dusted, and 3324 $cm.^2$ in experiment II, where the fruit were exposed to S^* vapor. The S^* covered a surface area of 2322 $cm.^2$ in experiment I (S^*-dusted) and only 63.5 $cm.^2$ (area of Petri dish) in experiment II (S^* vapor). The period of incubation was 68 hours in experiment I and only 30 hours (with the reaction bottle closed) in experiment II; thus both the area and the time of incubation were greater in experiment I. Since both of these factors would increase the amount of S^* vapor in experiment I, calculations

[1] The effect of increasing mass on the lowering of the vapor pressure of isotopic hydrogen molecules was discussed by KAMEN (33: 134, 135).

of the specific activities per cm.² per hour are given for the various products formed in experiment I (table 1) and experiment II (table 2), and their ratios are shown in table 3. Comparison of the specific activities of the H_2S^* formed per cm.² per hour shows that this gas was generated in experiment I at about one-fifth the rate in experiment II (table 3). Also the S^*O_2 was produced at about one-fifth the rate in experiment I as in experiment II (table 3). The S^*O_4 found in the peel in experiment I was about one-half as much as in experiment II. If the amounts of S^*O_4 in the peels and in the exudates from fruit in both experiments were known, the rates of formation of SO_4 might have been compared. As important as this point may be, however, contamination of the exudate from fruit in experiment I may have occurred because, in the intimate contact of the exudate with the radioactive S dust on the fruit, the same S-oxidizing enzymes may have been active in the exudate as in the peel. In experiment II, information on this point might have been obtained, but the exudate unfortunately was not assayed for S^*O_4.

TABLE 3

RATIO OF SPECIFIC ACTIVITIES OF RADIOACTIVE SULFUR PRODUCTS FORMED WHEN EUREKA LEMONS WERE DUSTED WITH ELEMENTAL RADIOACTIVE SULFUR OR EXPOSED TO VAPOR OF ELEMENTAL RADIOACTIVE SULFUR

Compounds	Ratio of specific activities (S*-dusted)	Ratio of specific activities (Exposed to S* vapor)	Compounds	Ratio of specific activities/cm²/hr (S*-dusted/ S* vapor)
H_2S^*/S^*O_2	7.14	5.79	H_2S^*/H_2S^*	0.219
H_2S^*/S^*O_4	1.33	3.06	S^*O_2/S^*O_2	0.178
S^*O_2/S^*O_4	0.19	0.53	S^*O_4/S^*O_4 (peel)	0.504

A photomicrograph of a radial section of the peel of a lemon used in experiment I is shown in figure 1, and a radioautograph of the same section is shown in figure 2. The greater darkening of the radioautograph in the tissue in the general region of the oil glands indicates a greater concentration of radioactive S in the outer regions of the peel. There is, however, also a region of increased darkness along the segment membrane on the interior side of the peel. Because of the type of histological treatment used in preparing the peel section (cf. "Material and methods"), it is thought that the radioactive S must have been synthesized into the peel proteins, in order to remain in the section. This conclusion was reached on the basis of the following reasoning. It seems probable that the proteins of the peel would be precipitated at the pH 3.5 of the killing fluid as well as by the 50% ethanol which the killing fluid contained.[2] Pectins also would probably be precipitated at this point, while such water-soluble constituents as sugars and organic acids and acetates of the cations would be lost. On washing with water the water-soluble materials already mentioned plus the anions, such as SO_4, SO_3, NO_3, etc., would be lost. The oils in the peel would likely be lost in the butyl alcohol treatments. It seems

[2] Since the completion of this paper additional work by us has shown that the acid-soluble protein was extracted, leaving only the alkali-soluble protein which was the more feebly labeled of the two.

probable, then, that of the insoluble constituents remaining, there are the cellulose walls and pectins which do not normally contain S, and the proteins which do. HARRISON *et al.* (**29**) published radioautographs of wheat leaves and kernels containing radioactive S which showed a similar diffuse distribution which they believed was largely contained in the applied as a dust when judged on the basis of the specific activity of the BaS^*O_4. On the other hand, when lemons were exposed to S* vapor in very limited quantities, some H_2S was produced which was not derived from the radioactive S. This is suggested by the low specific activity of the BaS^*O_4 derived from the H_2S^* produced. Though the ad-

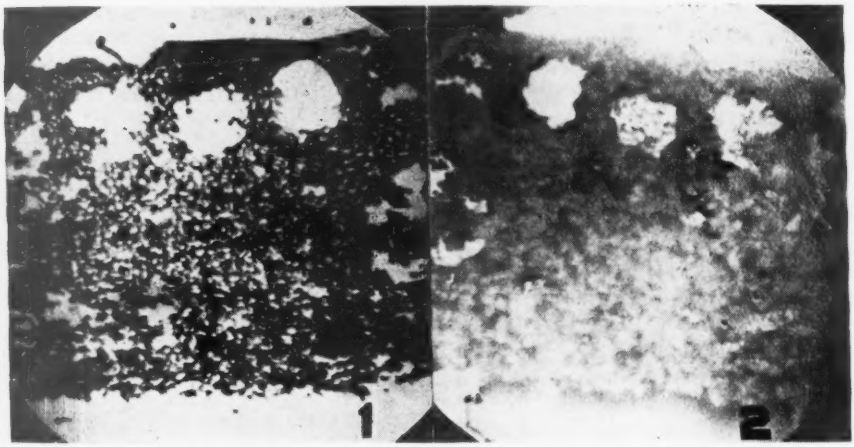

FIG. 1.—Photomicrograph (×20) of radial section 15 μ thick of peel of Eureka lemon dusted with ground 325-mesh radioactive elemental S and incubated at 106° F. for 68 hours.

FIG. 2.—Photomicrograph (×20) of radioautograph of section shown in Fig. 1. Right-hand side of fig. 2 coincides with left-hand side of fig. 1. Note darkening caused by greater concentration of radioactive sulfur on outer surface of peel (*upper side*), gradual diminution at greater depth in albedo, and concentration at segment membrane (*lower edge*). Because of killing with formalin-acetic-acid–alcohol mixture, radioautograph indicates incorporation of radioactive S in protein of peel. Vacant spaces at upper edge of figs. 1 and 2 are oil glands.

protein. Radioautographs (**6**) of human skin and pig skin exposed to S*-labeled mustard gas show a somewhat similar distribution of radioactive S which was concentrated in the epidermis, corium, sebaceous glands, hair follicles, and subcutaneous fibrous tissue.

Discussion

The H_2S^* produced by S*-dusted lemons incubated at 106° F. appears to be derived entirely from the radioactive S

ditive effect of the difference in mass of S^{35} and S^{32} under the three conditions listed earlier possibly could account for this discrepancy when the amount of S^{35} available to the fruit was very small, it is suggested that H_2S in very small amounts may have been produced by the fruit before sufficient radioactive S vapor was available for H_2S^* formation. This view is supported by the fact that if the total S* which vaporized from the Petri dish was instantaneously available to the

fruit, the concentration of S* vapor would not have been greater than 1.48 p.p.m. This view is further supported by the following facts: (*a*) THOMAS and HENDRICKS (**68**) have shown that H_2S is formed by mild alkaline hydrolysis of alfalfa leaves; (*b*) loss of water by leaf tissue is accompanied by a decrease in protein-S (**53, 54**); (*c*) citrus fruit loses water on incubation at warm atmospheric temperatures (**80**); and (*d*) occasionally citrus fruit, although not treated with S, will yield about 1 μgm. of H_2S (**79**).

In the S*-dusted fruit the specific activity of the BaS^*O_4 formed from the S^*O_2 evolved was 14.4% of the specific activity of the S* applied. The phosphate-borate buffer solution used to absorb the S^*O_2 in the H_2S^* which passed through it to the next gas absorption bottle has been shown to separate these two gases effectively (**68**). The BaS^*O_4 obtained from the S^*O_2 was redissolved after the first counting, reprecipitated, and recounted without showing a significantly different count. It thus appears that some radioactive SO_2 may have been formed and that the radioactivity of the compounds in the phosphate-borate buffer was not due to a small residual amount of H_2S^* in the buffer. In this experiment (experiment I) the ratio of specific activities of H_2S^* to S^*O_2 was 7.14 (table 3). In the experiment in which fruit was treated with S* vapor (experiment II), the ratio of the specific activities of H_2S^*/S^*O_2 was 5.79 (table 3). Former experiments, in which S^{32} was used, on the average gave a ratio of H_2S/SO_2 of about 4.0 at 105° F. (**79**). This comparatively close agreement in the ratios suggests that radioactive S^*O_2 is formed by S^{35}-treated fruit when incubated at warm air temperatures.

The specific activity of the S^*O_4 produced in the peel of S*-dusted fruit was about 77% of the activity of the S* applied, indicating that a substantial amount of the S^*O_4 formed was derived from the S* dust. This corresponded to a S* content in the peel of 0.136% and 0.141% for duplicate analyses. The average of a large number of SO_4 analyses on the peel of non-S-dusted fruit incubated 3–17 hours was about 0.1% S (**79**). An increase in the actual S* content of the S*-dusted fruit is indicated. About 82% of the activity of the S^*O_4 which was found in the exudate was derived from the S* dust.

In the fruit exposed to S* vapor the specific activity of the S^*O_4 was only 1.85% of the specific activity of the S* applied and indicates the preponderance of inactive SO_4. The S content of the peel determined as SO_4 was 0.274%; this is an unusually high value in the range of lemons treated with H_2S (**79**: table 17). Such a high S content of the peel indicates some extraordinary treatment of the tree and may account in part for the quantitative differences in the reactions of the S*-vapor treated fruit as compared with the S*-dusted ones.

The effects of limiting the surface area of the S and of the time of incubation on the total S volatilized are clearly evident on comparing the counts per second representing the total activity of the compounds formed in each experiment (table 4). Amounts of elemental S vapor as minute as those used in our Petri dish experiment appear to have been employed in other experiments designed to test the effect of elemental S vapor on living organisms. Negative results and the conclusions drawn from them should be viewed critically, considering our results, and especially so in such cases where the amount of S vapor formed has

not been determined. This experiment adds support to NORTHRUP's belief (**51**), concerning three alternative reactions of mustard gas with thiol enzymes, that the first molecule of gas coming in contact with the enzyme does not inactivate it. It will be noted that the specific activities of the compounds formed in the S*-vapor-treated fruit range around a tenth of the specific activities of the S*-dusted fruit (tables 1, 2). But when correction is made for difference in area of the evaporating surface of the S and for the exposure time, the S*-dusted fruit compounds had lower specific activities (tables 1, 2, 3).

actions with protein groups such as carboxyl, amino, tyrosine, tyrosine phenol, trytophane indol, histidine, imidazole, arginine, guanido, aliphatic hydroxyl of serine, threonine, hydroxy-proline, and thiomethyl ether linkage of methionine (**51**), a number of which react with mustard gas. But no attempt has been made to identify such compounds. A number of papers report the dismutation of thiol compounds yielding one or more of the same or similar products (**23, 39, 61, 68**).

TABLE 4

COMPARISON OF TOTAL ACTIVITIES IN COUNTS PER SECOND OF PRODUCTS FORMED BY DUSTING LEMONS WITH RADIOACTIVE ELEMENTAL SULFUR AND BY EXPOSING LEMONS TO VAPORS OF ELEMENTAL RADIOACTIVE SULFUR, WITH TOTAL ACTIVITIES OF RADIOACTIVE SULFUR VAPORS FORMED. CORRECTED FOR BACKGROUND, GEOMETRY, AND SELF-ABSORPTION

Product formed	Sulfur*-dusted		
H_2S^*	2.07×10^3	S* applied to fruit	5.03×10^5
S^*O_2	8.66×10^1	S* not vaporized	3.26×10^5
S^*O_4 (peel)	7.36×10^3	S* vaporized	1.77×10^5
S^*O_4 (exudate)	1.74×10^3	S* vaporized less total cts. in S* products	6.4×10^9
Total	1.13×10^4		

Product formed	Sulfur* vapor		
H_2S^*	1.70×10^1	S* vaporized	2.14×10^3
S^*O_2	2.06×10^9	S* vaporized less total cts. in S* products	1.52×10^3
S^*O_4 (peel)	6.00×10^2		
Total	6.19×10^2		

It has been shown that lemon fruit dusted with elemental S and incubated at 105° F. or above yields H_2S, SO_2, SO_4, NH_3, and oxalic acid (**79**). Also, it has been shown that lemons incubated in H_2S yield SO_2 and SO_4 and that, when incubated in SO_2, they yield SO_4 (**79**). In these gases other gaseous compounds may be formed as in the case of elemental S, and others not yet identified, from re-

TARVER and SCHMIDT (**66**) have shown that the addition of S* to glutathione and cysteine yields H_2S^* according to the equation

$$2RSH + S^* \longrightarrow RSSR + H_2S^*. \quad (1)$$

We have found that lemons dusted with S and incubated aerobically lose glutathione (GSH) (**79**); and we have shown that lemons dusted with S* give H_2S^* of high specific activity. It therefore appears that the H_2S^* is derived from glutathione and/or cysteine.

The mechanism involved in the formation of S^*O_4 apart from the inactive SO_4 appears to be that involved in the normal metabolism of S. If the H_2S^* formed in equation (1) is used as a starting point, it may be converted *in vitro* by cysteine desulfurase to radioactive cysteine in the presence of cysteine (**62**), which reaction has been shown to occur *in vivo* in rats (**20**). Although SMYTHE has expressly stated that the mechanism of the reaction is not elucidated by his study (**62**), the general reaction may be expressed thus

$$H_2S^* + 2RSH \xrightarrow{\text{cysteine desulfurase}} RSSR \quad (2)$$

$$RSSR + H_2S^* \xrightarrow{\text{cysteine desulfurase}} 2RS^*H \quad (3)$$

According to the rather well-fortified MEDES' theory (**47**), SO_4 formation could take place by two routes. Using RS*H as a starting material, one could expect S^*O_4 formation through the following mechanism:

$$RS^*H \xrightarrow{\text{cytochrome oxidase}} RS^*SR, \quad (4a)$$

or

$$RS^*H \xrightarrow{\text{cysteine oxidase (a)}} RS^*OH \quad (4b)$$

$$RS^*SR \longrightarrow RS^*OH + RSH \quad (5a)$$

$$RS^*OH \longrightarrow RS^*O_2H + RSH \quad (6)$$

$$RS^*O_2H \xrightarrow{\text{sulphinic acid oxidase}} S^*O_4 \quad (7)$$

This general course of events receives a degree of validation from the synthesis of protein* from S^*O_4 and the reconversion of S^*O_4 from protein* (**69**) and from the fact that we have obtained radioautographs of the peel of lemons dusted with radioactive S, suggesting the presence of S*-labeled protein.

The probable mechanism involved in the formation of the SO_2 is much less clear. Although SO_2 was recovered from cystine on alkaline hydrolysis of alfalfa leaves (**68**), SO_2 as such, in general metabolic studies, has not been reported. It tentatively may be suggested that the SO_2 arises from cystine and/or methionine. MEDES and FLOYD have shown that cystine oxidase (same as cysteine oxidase) converts cystine to cysteic acid (**47**). Perhaps the cysteic acid so formed may be transmuted, giving SO_2. It appears, however, that the oxidation of methionine results in disulfides (**55**) and that, if these are dismuted, then further oxidized, SO_2 might result. The fact, also, that the S^*O_2 has such a low specific activity suggests the destruction of some compound containing it. The destruction of methionine by selenium (**21**) and the "safening" effect of additions of methionine to selenate suggest that the destruction of methionine may occur in sulfur injury to citrus fruit with the evolution of SO_2.

An accounting of the activity of the S* applied to the fruit as a dust and as a vapor and the total activity found in the products formed by the fruit are shown in table 4. Only 6.4 cts/sec were unaccounted for in the products of the S*-dusted fruit. But 1500 cts/sec were unaccounted for in the compounds formed by the fruit exposed to the S* vapor. This amount of activity was probably adsorbed to the walls of the glass apparatus.

Summary

1. The S metabolism of citrus fruit—judged on its reaction with elemental S, in which H_2S and SO_2 gases and SO_4 are formed, its reactions with H_2S^* gas to give SO_2 and SO_4, and its reaction with SO_2 to give SO_4—exhibits many points of similarity with that of organisms ranging from lower animals to man and bacteria to angiosperms.

2. By the use of radioactive S (S^{35}) it was determined that a very large proportion of the S in the H_2S formed when lem-

ons are dusted with elemental S and incubated at warm atmospheric temperatures (106° F.) is derived from the S applied. The SO_4 formed in the fruit peel may be derived largely from the S applied if relatively large amounts of S are applied, but the SO_2 formed is derived largely from a source within the fruit.

3. Elemental S vapor penetrates lemons, producing compounds similar to those produced by fruit in contact with elemental S. The area of the S layer from which vaporization occurs and the length of exposure to warm temperatures (106° F.) influence the amount of S products formed by lemons.

4. Radioautographs of the peel of lemons dusted with radioactive S and incubated at 106° F. suggest the incorporation of the S into the tissue proteins.

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THE CHLOROPHYLL MUTANTS OF MAIZE[1]

DREW SCHWARTZ

Introduction

The chlorophyll mutants of *Zea mays* have been studied extensively by geneticists. They are useful in the study of Mendelian inheritance, since the mutant plants can usually be recognized early in the seedling stage. Some of the mutants, such as iojap (**15, 20**), are used as tools for the study of cytoplasmic inheritance. In view of the importance of these mutants in genetical studies, and their probable value in the attack on the problem of photosynthesis as pointed out by DEMEREC (**3**), it is surprising that so few investigations have been devoted to a study of the plastids and plastid pigments present in these mutants.

RANDOLPH (**19**) showed that, contrary to the findings of MILES (**17**), white mutant plants do contain plastids. These plastids are minute because of the failure of the proplastids to develop normally. ZIRKLE (**24, 25**) confirmed RANDOLPH's observations that the proplastids of the various mutant chlorophyll types are normal and found that the divergent phenotypes are due to (*a*) a delayed development of the plastid, (*b*) a stoppage of development, or (*c*) a stoppage of development followed by degeneration. EYSTER (**10**) found giant plastids in albino plants and thus concluded that there is no correlation between plastid size and the amount of pigment developed in the chloroplasts. In work with barley, however, EULER and co-workers (**8, 9**) and GUSTAFSSON (**13**) found a definite parallelism between plastid size and pigment formation.

There are a large number of chlorophyll mutants of maize, many of which have been accurately located on the chromosome maps. EMERSON *et al.* (**7**) listed twelve whites, seven luteus, ten pale greens, three yellow-greens, four goldens, and twenty virescents. Because of the large amount of work involved, many new mutants have not been tested to determine whether they involve new loci or are allelic to the mutants found previously.

All the white and luteus mutant plants are lethal. Since they are unable to carry on photosynthesis, they die in the seedling stage after having exhausted the food available in the endosperm. The virescents are characterized by a retardation of chlorophyll development. Greening may proceed from either the tip or the base of the leaf. Usually the fifth or sixth leaf emerges fully green, although some virescents may show striping when mature. Viability depends on the rate of greening. Plants which turn green slowly are usually lethal (**1, 6, 18**).

In the case of the pale greens there does not appear to be a close relationship between viability and the amount of chlorophyll synthesized by the plant. DEMEREC (**2**) found that Xantha 2, a pale-green mutant having only 10% of normal chlorophyll content, can be grown to maturity, while pale-green 5 with 80% of normal chlorophyll content is lethal. He thought that the lethal pale greens might contain different chlorophyll pigments which cannot function in photosynthesis. INMAN and BLAKESLEE (**14**)

[1] The work on which this paper is based was done with the aid of a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

have, in fact, reported a pale-green mutant of *Datura* whose chlorophylls were spectroscopically different from chlorophylls *a* and *b*.

The purpose of this investigation was to study the relationship between the size and morphology of the chloroplasts and the kinds and amounts of plastid pigments found in the mutant plants.

Material and methods

The chromatographic adsorption technique was used to separate and isolate the pigments. Fresh leaves were macerated with a mortar and pestle in a solution of petroleum ether and benzene (9 : 1) to which a few milliliters of methyl alcohol were added. After extraction the solution was washed four or five times with distilled water to remove the methyl alcohol and passed through adsorption columns $\frac{1}{2}$ inch in diameter and about 10 inches high. The latter were packed with activated alumina in the lower third, calcium carbonate in the middle third, and XXXX confectioner's sugar in the upper third (**23**).

In the development of the chromatogram the carotenes pass through the top two layers and are adsorbed on the alumina. The xanthophylls band out on the calcium carbonate, and two green bands form on the sugar: an upper yellow-green band of chlorophyll *b* and a lower blue-green band of chlorophyll *a*. After all the pigments had been adsorbed, the column was washed with a 9 : 1 solution of petroleum ether and benzene. The bands separated well, leaving wide, clear spaces between them.

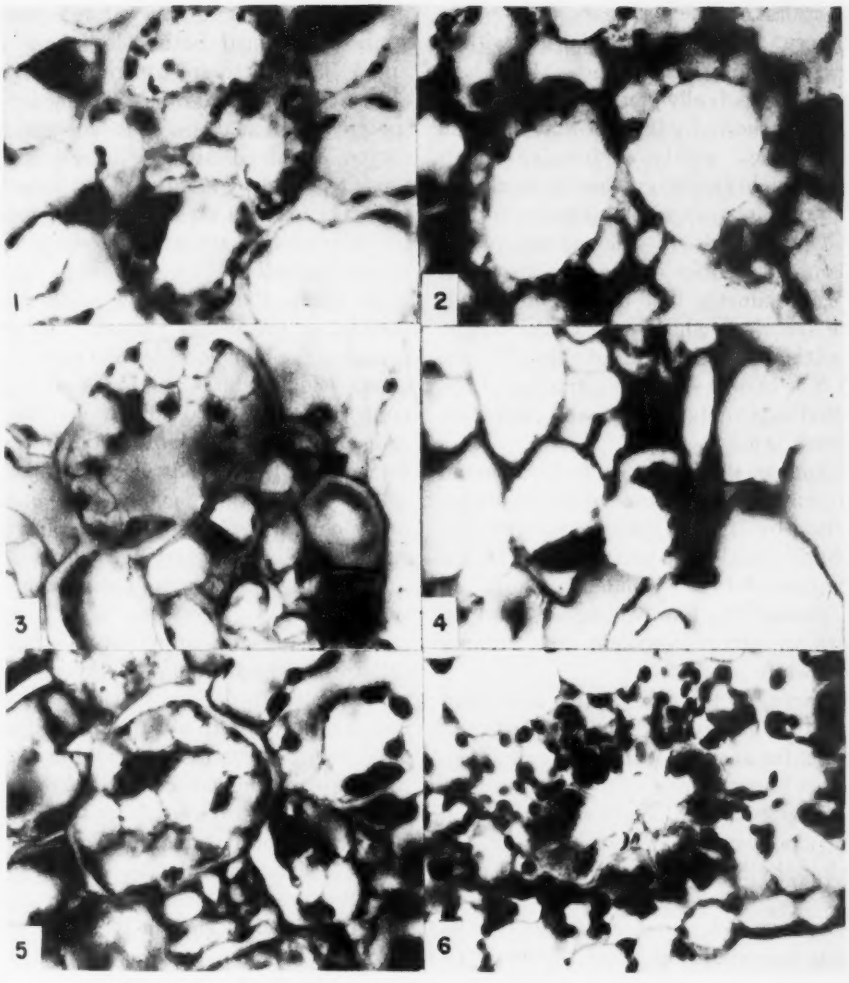
Further to identify the two green pigments involved, their absorption spectra were obtained by means of a Beckman spectrophotometer. To accomplish this, the column was pushed out of the glass tubing and cut into sections separating the green bands. These sections were then trimmed so that only the pigment adsorbed in the central part of the column was used, thus eliminating the possibility of contamination by other pigments during the process of removing the column from the glass tubing. These sections were immediately stirred into ether. When the sugar settled, the ether-pigment solution was decanted, filtered, and stored at 0° C. for not more than 5 hours before the spectrophotometric analysis was made.

To study the chloroplasts of the mutant plants, leaves were cut and immediately fixed in CRAF solution. They were imbedded in paraffin and sectioned at 12 μ. The material was stained with safranin-fast green or hematoxylin. Measurements of plastids were made with an ocular micrometer.

Results and discussion

WHITES.—Two types of so-called "white" mutants were studied, the pure whites and the cream-colored whites. The pure whites lack all chloroplast pigments, while the cream whites were found to contain a very small amount of both carotene and xanthophyll but to lack the chlorophylls. Most of the whites are of the latter type.

Cytological observation revealed that the mesophyll plastids are minute, less than 1 μ in diameter. In many whites the plastids of the parenchyma sheath which incloses the vascular bundle of the leaf are large, 4–8 μ, but distinctly abnormal, bearing little resemblance to the normal plastid. They consist of distended membranes inclosing small deeply staining bodies and give the appearance of small cells within the bundle-sheath cell (fig. 1). These are probably identical with the abnormal plastids that RANDOLPH found in his "Mendelian white" strain. He de-



FIGS. 1–6.—Cross sections of leaves of *Zea mays*. Figs. 1–5, ×720, Fig. 6, ×414. Fig. 1, from white mutant, showing abnormal plastids in parenchyma-sheath cells. Fig. 2, from luteus leaf. "Bubble-like" structures in parenchyma-sheath cells are clearly visible. Note that plastids are larger than those in fig. 1. Many luteus mutants contain fewer plastids than normal green plants. Fig. 3, from virescent leaf. Each immature plastid of parenchyma sheath is located within one of "bubbles" formed by apparent distention of semipermeable membrane which surrounds plastid. Fig. 4, from virescent leaf plasmolyzed in 0.3 *M* sucrose solution. Compare with fig. 3. Plasmolysis caused membrane to shrink around inclosed plastid. Fig. 5, from leaf of pale green mutant with abnormal plastids in parenchyma-sheath cells. Plastids in mesophyll cells are of normal size. Fig. 6, from normal leaf showing difference between mesophyll and parenchyma-sheath plastids.

scribed them as being colorless and having a darker irregular mass near the periphery, resembling nuclei each with a densely staining nucleolus. That these bodies are actually greatly modified plastids was shown by the fact that they still retain their ability of forming starch granules when the cut leaf is immersed for 30 hours in an 0.8 *M* sucrose solution.

The plastids of the normal green plants studied for comparison are about 6 μ in diameter. The mesophyll plastids are spherical, while those of the bundle sheath appear as flattened ellipsoids (fig. 6). The bundle-sheath plastids have been described in detail by RHOADES and CARVALHO (**21**).

None of the whites studied was found to contain large normal plastids as reported by EYSTER (**10**). RANDOLPH (**19**) found normal green plastids at the tips of the leaves of his "Mendelian white." In all probability, however, he was working with an extreme virescent rather than a true white.

Hence, in the case of the white mutants, a positive correlation exists between the absence of the chlorophyll pigments and the failure of the plastids to develop normally. In this investigation no plants were found which lacked the chlorophylls but had normal-sized plastids.

LUTEUS.—A number of luteus mutants were studied in which the color varies widely from very pale to deep yellow. Chromatographic analysis of their pigments established that they all contain, in addition to the carotenoids, a small amount of chlorophyll. The deep yellow plants were found to contain more chlorophyll than the pale-yellow seedlings, indicating a relationship between chlorophyll and carotenoid production. In the pale-yellow plants the concentration of chlorophyll is so low that the green color is completely masked by the more abundant yellow pigments. Every luteus plant studied contained both chlorophylls *a* and *b*. That these green pigments actually are chlorophyll was confirmed by spectroscopic analysis. The absorption spectra obtained fit very closely with those reported by ZSCHEILE and COMAR (**26**). The maxima are at 660 and 430 mμ for chlorophyll *a* and at 645 and 455 mμ for chlorophyll *b*.

All plants that contain a substantial amount of the yellow pigments always contain some chlorophyll. Only the cream-colored whites possess the carotenoids but lack chlorophyll. In these cases, however, the concentration of carotenoids in the plant is very low. Thus, whereas the white genes completely block chlorophyll formation, the luteus genes only limit to varying degrees the total amount of chlorophyll which can be synthesized.

An obvious correlation between plastid size and pigment production in the luteus mutants does not exist. Plastid size varies from 1 to about 4.6 μ. Either large or small plastids are found in both the deep- and the pale-colored plants, although plastid size in each mutant type is fairly uniform (table 1). The plants were classified for color by comparison with RIDGWAY's color charts (**22**).

It is of interest that in these mutant plants some chlorophyll is formed by plastids as small as 1 μ. RANDOLPH, in his study of plastid development in normal green plants (**19**), found that chlorophyll is not present in the plastids until they have attained at least one-half of their full size, 3–4 μ.

In many of the luteus mutants the plastids are concentrated in the bundle-sheath cells with but few in the mesophyll, while in others the plastids are numerous in both tissues. As expected,

those plants in which plastids are limited mainly to the bundle sheath have their pigments concentrated in the same tissue.

Some of the luteus plants have their bundle-sheath plastids inclosed in an expanded membrane which closely resembles the abnormal structures found in the white plants (fig. 2).

One luteus mutant was found in which the formation of chlorophyll *b* is retarded. GOODWIN and OWENS (**11**) found that chlorophyll *b* follows the formation of chlorophyll *a* by about 3 hours when etiolated oat seedlings are exposed to light. In the case of the luteus mutant, chlorophyll *a* preceded the synthesis of chlorophyll *b* by 4 days when grown at 85° F. The formation of chlorophyll *b* is strongly influenced by temperature. At 62° F. the retardation extended over a period of 11–14 days. Chlorophyll *b* is first synthesized in the oldest tissue in the leaf, the tip, which then appears greener than the rest of the leaf. This mutant is being studied further in an effort to determine whether photosynthesis can be carried on by higher plants in the absence of chlorophyll *b*.

VIRESCENTS.—With the virescents a close correlation exists between the size of the chloroplasts and the amount of chlorophyll present. In the yellowish regions of the leaf the plastids are small, and the chlorophyll content is very low. As is the case with the luteus plants, both chlorophylls *a* and *b* are present. In proceeding along the leaf toward the green areas, the chlorophyll content increases concomitantly with plastid size, as was earlier found by RANDOLPH (**19**).

Cytological study of the plastids in the virescent plants provided strong support for the assumption that the bubble-like bodies observed in the white and luteus seedlings are closely associated with plastid development. Since they are also found in the bundle-sheath cells of the virescent seedlings, it was possible to follow their development from the yellow to the green regions of the leaf.

By means of sections made at close intervals along the virescent leaf, plastid development could be followed fairly easily. It was clear that each immature bundle-sheath plastid is located within one of the "bubbles" (fig. 3). As the plastid grows larger, it occupies more and more of the area within the extended membrane until it fills it completely and appears similar to the plastids of normal green plants. In general, this type of plastid development is found only in the bundle-sheath cells; one mutant, however, showed a similar development of the mesophyll plastids.

TABLE 1

COMPARISON OF PLASTID SIZE AND PLANT COLOR OF LUTEUS MUTANTS

Color of leaves*	Largest dimension of plastids in μ†
Pale mutants (low chlorophyll content):	
Straw yellow	1.0; 1.0
Amber yellow	3.5; 1.5; 3.5
Naphthalene yellow	1.0
Barium yellow	1.2–2.3; 2.3
Citron yellow	2.3–4.6‡; 2.3; 1.2; 1.0; 1.0
Pinard yellow	1.5; 2.8; 2.3
Picric yellow	4.6
Deep mutants (high chlorophyll content):	
Bright green-yellow	4.6
Light yellow-green	4.6; 2.3; 3.0

* Color comparison made with RIDGWAY's color charts (**22**).

† These figures represent average dimensions of eight to ten plastids; each figure represents a different strain.

‡ Large plastids are found in bundle-sheath cells only.

In an effort to learn more about the membranes which surround the immature plastids, some virescent leaves were plasmolyzed in a 0.3 *M* sucrose solution before being fixed in CRAF. When these leaves were sectioned and stained, it was

found that the large, bubble-like bodies had been tremendously reduced in size until they were not much larger than the undeveloped plastids which they inclosed (fig. 4). Thus, it appears as though these structures in the unplasmolyzed leaves were swelled with water.

One possible explanation of the "bubbles" and the fact that they are commonly found only in bundle-sheath cells may be that the semipermeable membrane surrounding the immature plastid is distended by water intake through osmosis. KNUDSON (16) found that "plastids exhibit definite osmotic properties and therefore must possess a semi-permeable membrane." The plastids swelled when he caused them to extrude from the cells into distilled water and enlarged until they resembled minute soap bubbles, with the chlorophyll bunched in a basal region. This is analogous to the condition encountered with the immature plastids within the bundle-sheath cells of the virescent leaves. GRANICK and PORTER (12) also found that plastids exhibited osmotic properties. Since the bundle-sheath cells immediately surround the vascular tissue, the diffusion pressure deficit of the sap in these cells must be lower than that of the adjacent mesophyll cells. DIXON (4, 5) has shown that this condition must exist to facilitate movement of water from the vascular tissues to the evaporating surface of the leaf. It may be that the diffusion pressure deficit of the bundle-sheath plastid is sufficiently higher than that of the cell sap to allow for the osmotic movement of water from the cell vacuole into the plastid. In the mesophyll cells the difference between the osmotic pressures of the cell sap and the plastid may not be great enough to cause water to be drawn from the cell vacuole into the plastids. Thus, while the bundle-sheath plastids remain small, the membranes surrounding them are greatly expanded, giving the appearance of bubbles. When they are plasmolyzed, water is drawn out, and the membrane shrinks and collapses around the immature plastid.

PALES GREENS.—The pale-green and yellow-green mutants are characterized by a low chlorophyll content of the plant. The plastids are large, varying in size from normal to slightly smaller than normal. All the pale greens studied, including lethal and viable mutant types, were found to contain both chlorophylls *a* and *b* as determined by spectroscopic analysis. Thus, it was established that the lethality of many of the pale greens cannot be accounted for by the absence of one of the chlorophylls or by a modified chlorophyll which cannot function in photosynthesis.

It is possible to distinguish cytologically between the viable and lethal pale greens. The bundle-sheath plastids of the viable mutants are normal in appearance, while those of the lethal plants studied lack the starch inclusions normally found and closely resemble normal mesophyll plastids.

One lethal mutant was found which had normal-sized mesophyll plastids, but every bundle-sheath cell contained only abnormal plastids similar to those described for white mutants (fig. 5). Thus, it seems that the degree of development of the mesophyll and bundle-sheath plastids is not always correlated.

The ch'orophyll mutants of maize form a complete series with all gradations from white to fully green plants. The pure whites at the bottom of the series are devoid of all pigments. Next come the cream whites, which contain some carotenoids but lack the chlorophylls. The luteus mutants follow, ranging from very pale-yellow plants, containing only a mi-

nute amount of chlorophyll, to the deep-yellow plants with considerably more chlorophyll. Beyond them lie the pale greens, and finally the fully green plants. The cream-colored whites can be differentiated from the pure whites by the fact that they contain some carotenoid pigments. The absence of the chlorophylls in the white plants distinguishes them from the luteus mutants. There are, however, no sharp dividing lines among the other classes, and many intermediate stages are found.

Summary

1. A detailed study of the white, luteus, virescent, and pale-green mutants of maize revealed that a close correlation exists between plastid size and chlorophyll development in all but the luteus plants.

2. Abnormal plastids in the bundle-sheath cells of the mutant plants were described and were thought to result from a distention by osmosis of the semipermeable membrane surrounding the immature plastids.

3. All the luteus plants studied were found to contain small amounts of chlorophylls *a* and *b*.

4. The chlorophylls were eliminated as the cause of the lethality of many pale greens, since both chlorophylls *a* and *b*, as determined by spectroscopic analysis, were found to be abundant in the lethal as well as in the viable mutants.

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EFFECT OF ALPHA-NAPHTHALENE ACETAMIDE UPON THE ANATOMICAL STRUCTURE OF COCKLEBUR GROWN IN A NUTRIENT MEDIUM DEFICIENT IN CALCIUM[1]

B. ESTHER STRUCKMEYER

Introduction

Information on the effects of growth substances in relation to mineral nutrition of plants is limited to a few papers on particular elements. A beneficial effect, as increased total growth, has been found from application of indole-3-acetic acid to water cultures of tomato and sunflower in incipient stages of zinc deficiency (6) and to cotton plants in early stages of boron deficiency (2). SKOK (5) reported that growth of bean plants in a calcium-deficient medium resulted in death of the plants within 52 days. MITCHELL (4) stated that one effect of alpha-naphthalene acetamide on the anatomical structure of bean plants was to stimulate an increase in cambial activity in the stem, resulting in the formation of a greater number of thick-walled xylem cells than were formed in the control.

The present paper deals with the effects of the application of alpha-naphthalene acetamide on the growth and the anatomical structure of stems of cocklebur grown in sand with complete and calcium-deficient nutrient solutions.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Material and methods

Seeds of cocklebur (*Xanthium echinatum* Murr.) were germinated in soil. Plants 6 inches in height were transferred to washed white quartz sand in glazed crocks and given approximately 500 ml. of Hoagland's nutrient solution (3) three times a week. They were watered on alternate days and the sand thoroughly leached once a week to maintain a more balanced solution. The greenhouse temperature for the first experiment was held at approximately 70° F.; for the second and third, 65° F.; and for the fourth, 75° F. Long-day photoperiod was maintained for all experiments so that the plants would remain in a vegetative state.

Half the plants in each experiment were given a complete nutrient medium; the others a nutrient medium without calcium. Half of each of these sets were treated with a $\frac{1}{2}\%$ concentration of al-

pha-naphthalene acetamide in lanolin, applied to the third internode and petiole of the leaf. Plants other than those used in these experiments were sprayed with a solution of the growth-regulator, and results similar to those with the compound in lanolin were obtained.

Symptoms of calcium deficiency were recorded at regular intervals. Samples were taken from the second, fourth, sixth, and eighth internodes of plants grown under the four sets of conditions. The stems were fixed in CRAF (Randolf's Modified Navashin Fluid) for staining; and in absolute alcohol and formalin for microincineration and study of the ash residue (7). The material fixed with CRAF was dehydrated in *n*-butanol and imbedded in paraffin. Stem sections 12 μ in thickness were cut on a sliding microtome. Iron-alum-hematoxylin and safranin, or fast-green and safranin, were the stain combinations used for anatomical examinations. Incinerated sections were examined for distribution of the mineral ash and chemically tested for the presence of calcium (1).

Results and discussion

GROSS EFFECTS.—In the spring of 1947 seedlings of cocklebur were planted in white quartz sand. Thirty-eight days later the plants deprived of calcium and not treated with the acetamide showed severe deficiency symptoms, such as short upper internodes, darkening of the leaf margins, downward cupping of the leaves, brown areas on the surfaces of the leaves, and drying of the stem tips. The untreated plants given a complete nutrient supply appeared normal; those deprived of calcium but treated with alpha-naphthalene acetamide made an almost normal growth, except that the leaves were smaller; those given an adequate calcium supply and treated with the acetamide showed a normal growth. For this experiment the temperature was maintained at approximately 70° F.

The experiment was repeated in the winter of 1948 in a greenhouse with a constant temperature of 65° F. After 14 days brown areas were evident on the surfaces of the younger leaves near the stem tips of untreated plants not supplied with calcium; the mature leaves had black margins, and downward cupping was apparent. Thirteen days later (27 days after planting) the deficiency symptoms had become much more pronounced. Plants grown without a calcium supply but treated with alpha-naphthalene acetamide were also starting to show symptoms of calcium deficiency. After 50 days the experiment was terminated. At this time the stem tips had dried on the untreated, calcium-deficient plants; the upper leaves were severely curled, deformed, and dry. Plants treated with alpha-naphthalene acetamide and deprived of calcium likewise showed a cupping of the leaves and short internodes; the stem tips were still green, and, although the deficiency symptoms were conspicuous, they were not so severe as in plants that did not receive the acetamide. This experiment was again repeated, and similar results were obtained. Thus, at a temperature of 65° F. treatment with alpha-naphthalene acetamide was not strikingly effective in delaying symptoms of calcium deficiency, in contrast to the results of the first experiment which was conducted at a warmer temperature.

An experiment using the same procedures was set up in the greenhouse at a constant temperature of 75° F. Deficiency symptoms were apparent after 16 days on untreated plants deprived of calcium (fig. 1); the margins on the upper leaves were turning dark and cupping

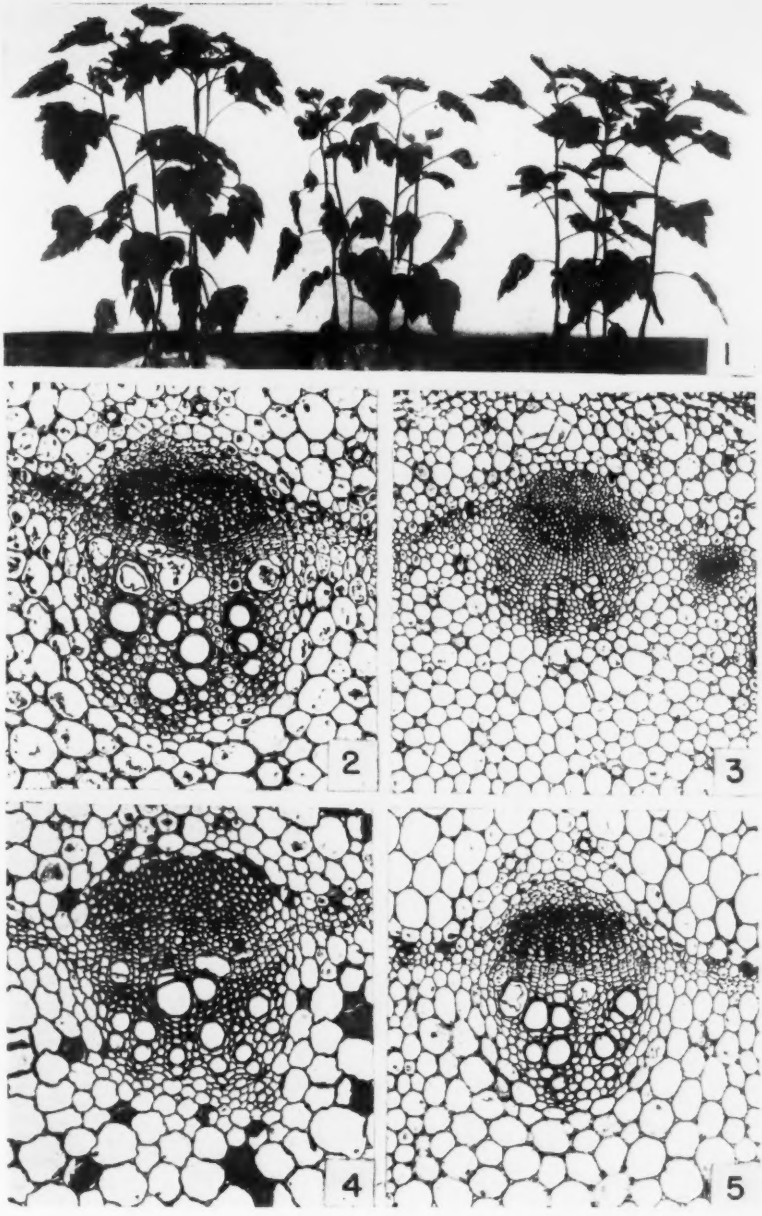
downward. Several days later these symptoms had extended to the lower parts of the plants; the stem tips had turned yellow, and an increasing number of leaves were cupped downward and showed brown areas. After 46 days these plants grown without calcium had shriveled and dried except for a few bottom leaves. They were then harvested and sampled for anatomical studies. Seventy-four days from the start of the treatment the plants grown without calcium and treated with alpha-naphthalene acetamide showed a slight cupping and browning of the upper leaves. Thus, at a temperature of 75° F. alpha-naphthalene acetamide was so effective that symptoms of calcium deficiency in treated plants did not appear until 20 days after the untreated, calcium-deficient plants were dead (table 1).

ANATOMICAL RESPONSES.—In the first experiment a striking effect of calcium deprivation on the anatomical structure of the stem was a decrease in the lignification of cells. MITCHELL (5) reported that alpha-naphthalene acetamide brought about an increase in cambial activity, resulting in greater secondary thickening and lignification. With calcium-deficient plants the effect on the anatomical structure was quite the opposite.

The fourth internode of untreated plants grown at 75° F. without calcium (fig. 3) differed from that of the normal plants (fig. 2) in that the cells of the stem were generally smaller and thinner-walled; this was particularly conspicuous for the xylem vessels, xylem parenchyma cells, and pericyclic fibers. Thirty-eight days from the start of the treatments the anatomical structure of the fourth internode of plants grown without calcium, but treated with alpha-naphthalene acetamide (fig. 5), was similar to untreated plants given a complete nutrient solution (fig. 2); the vascular bundles were somewhat smaller than in the normal stem, but the size of the cells and the degree of lignification were similar. On the other hand, the stems of treated plants given complete nutrient solution (fig. 4) showed an active cambium and a greater number of lignified xylem elements and pericyclic fibers than in the normal plant.

The eighth internode of the normal plant showed considerable secondary thickening. At the same level the vascular bundles of untreated plants deprived of calcium were smaller and the cells of the xylem region were thinner-walled; the xylem vessels displayed a lack of rigidity, as frequently the cell walls were in stages of collapse; cambial activity was apparent, but differentiation of normal vascular tissue appeared to be inhibited; the walls of the pericyclic fibers were less lignified than in the normal plants. The eighth internode of treated plants given a complete nutrient solution showed a prominent cambial zone with the formation of a greater number of thick-walled cells than in the normal plant; the xylem elements consisted primarily of tracheids; fewer vessels were present; and thickening of the walls of the cells in the interfasicular region was conspicuous. The structure of the eighth internode of treated plants grown without calcium resembled stems of treated plants given a complete solution; there was active cambial activity with excessive xylem formation.

In the second experiment the structure of the stems of plants grown at the cooler temperature of 65° F. was examined (figs. 6–9). The cells of the fourth internode of untreated plants given complete nutrient (fig. 6) appeared normal, like those grown at 75° F. (fig. 2). A similar region in the stem of untreated

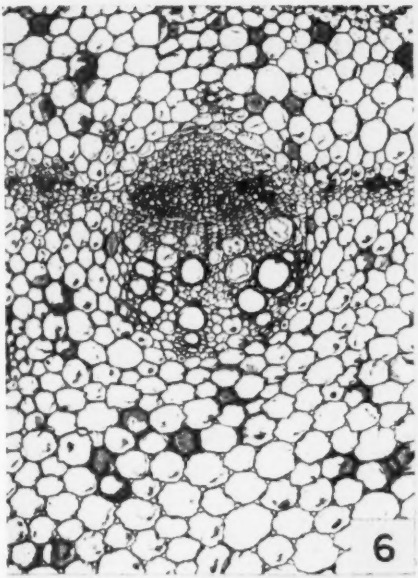


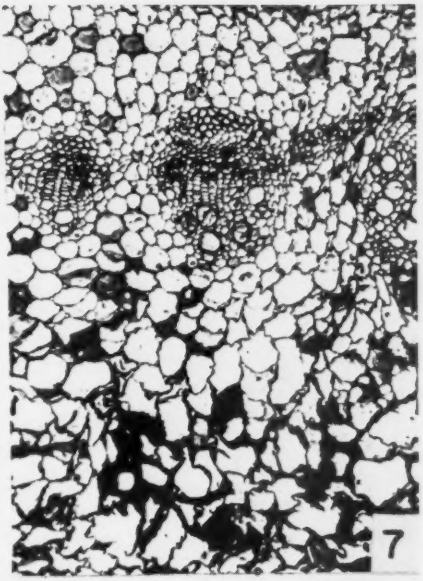
FIGS. 1–5.—Fig. 1, plants of cocklebur grown at temperature of 75° F. Photographed and sampled for anatomical examination 43 days after start of experiment. *Left*, untreated plant given complete nutrient solution (control). *Center*, untreated plant grown with calcium-deficient culture medium. *Right*, plant grown without calcium and treated with alpha-naphthalene acetamide. Figs. 2–5, cross sections of fourth internodes of vegetative plants of cocklebur grown at temperature of 75° F. Fig. 2, normal structure of plant grown with complete nutrient solution. Fig. 3, abnormal structure in plant grown without calcium; cells smaller and less lignification of these cells apparent. Fig. 4, structure of plant grown with complete nutrient solution and treated with alpha-naphthalene acetamide; active cambium, xylem elements and pericyclic fibers thicker-walled than in fourth internode of untreated plants grown with complete nutrient solution. Fig. 5, structure of plant grown without calcium but treated with alpha-naphthalene acetamide; vascular bundle slightly smaller than in normal plant; cell size and cell-wall thickening more nearly resembles structure of normal plant than plants from other two treatments. At this temperature symptoms of calcium deficiency were delayed by treatment with alpha-naphthalene acetamide.

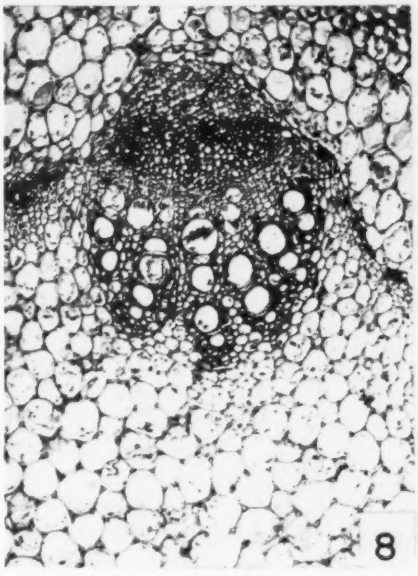
TABLE 1

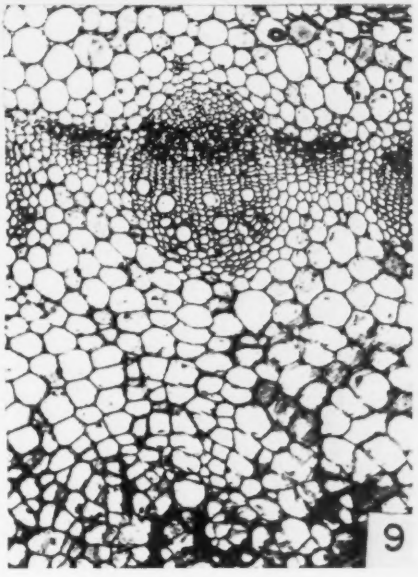
CALCIUM-DEFICIENCY SYMPTOMS OF COCKLEBUR PLANTS

Treatment		External symptoms of calcium deficiency				Anatomical abnormalities resulting from calcium deficiency			Mineral disbribution (relative amount of mineral residue)
		Blackening of margins and spotting of upper leaves	Downward cupping of leaves	Necrosis of apical meristem	Abnormal development of lower leaves	Cell size	Lignification of cells	Necrosis of tissue	
−Calcium	65° F.	14 days	20 days	27 days	38 days	Cells of vascular bundles smaller	All cells thinner-walled, especially xylem tissue	Necrosis in phloem and pith	+
−Alpha-naphthalene acetamide	75° F.	16 days	30 days	35 days	46 days	All cells smaller	Cells thinner-walled. Conspicuous in xylem tissue and in pericyclic fibers	Necrosis in phloem and pith	+
+Calcium	65° F.	.....	.....	.....	.....	Normal	Normal	None	++++
−Alpha-naphthalene acetamide	75° F.	.....	.....	.....	.....	Normal	Normal	None	++++
−Calcium	65° F.	32 days	44 days	Still normal 51 days after treatment	Still green 51 days after treatment	Cells slightly smaller than control; vascular bundles smaller	Thinner-walled xylem tissue	Necrosis in pith; not so severe as in untreated, calcium-deficient plant	+
+Alpha-naphthalene acetamide	75° F.	74 days	74 days (sampled)	.....	.....	Vascular bundles slightly smaller; cell size normal	Normal	.....	+++
+Calcium	65° F.	.....	.....	.....	.....	Cell size generally same as control	Increased lignification of xylem cells	.....	+++++
+Alpha-naphthalene acetamide	75° F.	.....	.....	.....	.....	Vascular bundles larger; cell size normal	Increased lignification of xylem cells, pericyclic fibers; greater cambial activity	.....	+++++









FIGS. 6–9.—Cross sections of fourth internodes of vegetative plants grown at temperature of 65° F. Fig. 6, normal structure in untreated plant grown with complete nutrient solution. Fig. 7, abnormal structure in untreated plant grown without calcium; vascular bundle smaller; fewer lignified cells than in normal plant; necrosis apparent in pith and extending to perimedullary zone. Fig. 8, structure of plant grown with complete nutrient solution and treated with alpha-naphthalene acetamide; xylem cells surrounding xylem vessels thicker-walled than in normal plant; at this temperature alpha-naphthalene acetamide not so effective in inducing lignification of cells as at temperature of 75° F. Fig. 9, abnormal structure of stem from plant grown without calcium but treated with alpha-naphthalene acetamide; structure more nearly resembles fig. 8; vascular bundle small with thinner-walled cells; indications of necrosis in region of pith. At this temperature alpha-naphthalene acetamide only slightly effective in delaying symptoms of calcium deficiency.

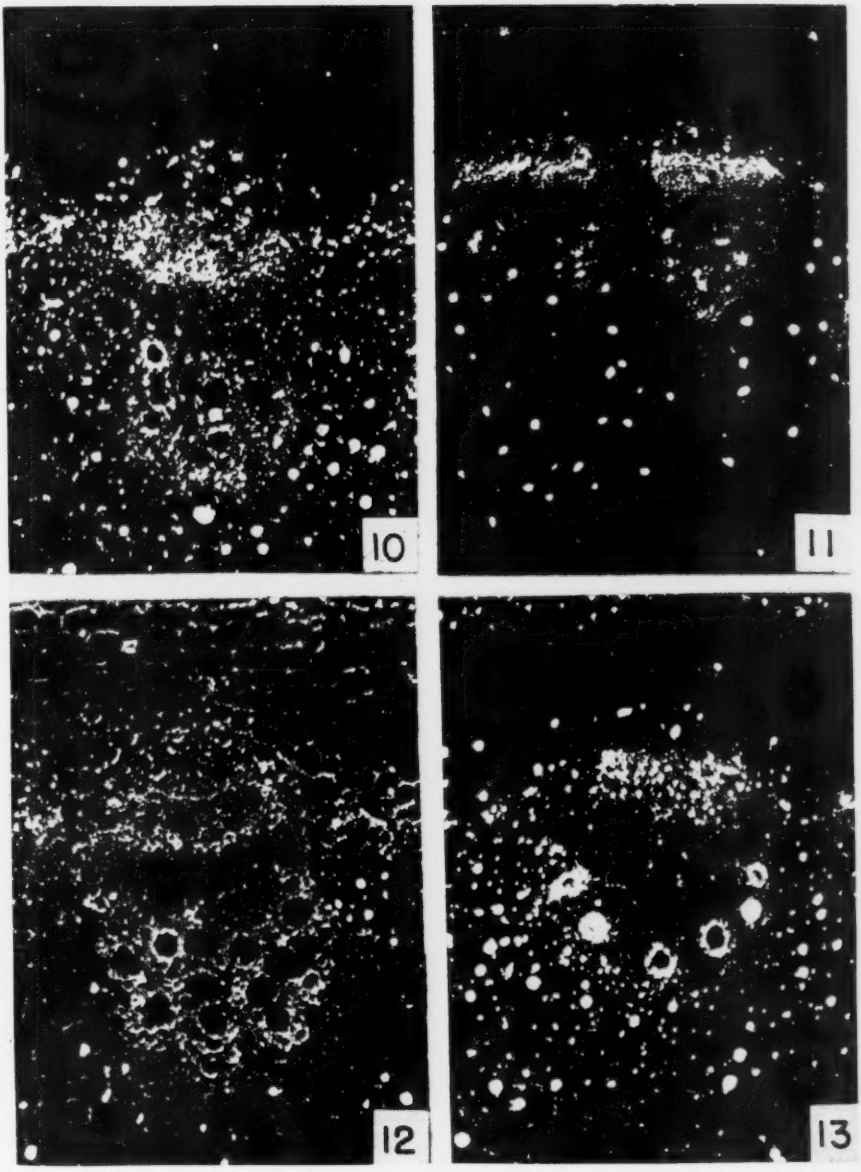
plants grown without calcium (fig. 7) showed cellular disorganization; the bundles were small, and a limited amount of secondary thickening had occurred; the parenchyma cells, especially those in the perimedullary region and the pith, were in stages of collapse; the center of the pith showed darkly stained areas, and necrosis was apparent in these regions. Alpha-naphthalene acetamide did not have so great an effect in delaying symptoms of calcium deficiency at this temperature as at the higher temperatures. The stems of treated plants grown without calcium (fig. 9) had smaller bundles and fewer vascular elements than the normal stem (fig. 6); near the center of the pith the cells were somewhat collapsed, giving the appearance of necrotic areas. Cellular abnormalities were not so severe in these plants as in the untreated plants deprived of calcium (fig. 7). A third experiment was conducted at this temperature with the same general results.

In the fourth experiment, a repetition of experiment I, symptoms of calcium-deficiency were again delayed by treatment with alpha-naphthalene acetamide. Also, the characteristic features of the anatomical structure described for the different sets of conditions in the first experiment were again obtained.

In addition to those stained, sections of the fourth internodes of stems grown under the four sets of conditions were prepared for microincineration (7). After incineration the sections were photographed for a record of the distribution of ash (figs. 10–13). The greatest amount of ash was present in stems of plants grown with complete nutrient solution and treated with alpha-naphthalene acetamide (fig. 12); stems of untreated plants grown with a normal calcium supply (fig. 10) showed the next greatest amount; stems of treated, calcium-deficient plants (fig. 13) had mineral residue closely resembling that of the normal plants; and stems of untreated, calcium-deficient plants (fig. 11) showed considerably less residue than the others. The mineral residue had various forms: it occurred as slender rods, particles arranged at random, in a continuous chain, or in irregular masses. Microchemical tests with 2% sulfuric acid gave positive tests for calcium in sections from the four treatments. Sulfuric acid caused the disappearance of the rodlike structures but not of other particles. It is perhaps possible that these may have contained the calcium which was dissolved and re-precipitated as calcium sulfate. No attempt was made to determine quantitatively the amount of calcium present.

Symptoms of calcium deficiency in cocklebur appeared considerably earlier than deficiency symptoms of other elements (K, P, Mg) in cultures grown at the same time and deficient in these elements. It is known that calcium is relatively immobile, so that little re-export occurs from one tissue to another. The present study showed that plants deficient in calcium had small thin-walled cells, and that necrosis of some of these thin-walled cells followed. If alpha-naphthalene acetamide was applied to the plants growing without adequate calcium, deficiency symptoms were delayed and the cells were larger and thicker-walled. Microincineration studies also revealed that a greater amount of mineral residue was present in plants grown without calcium and treated with the acetamide than in similar untreated plants. Some calcium was present, however, in all sections, regardless of treatment.

The exact role of alpha-naphthalene acetamide in delaying symptoms of cal-



FIGS. 10–13.—Mineral residues of cross sections of fourth internodes of incinerated stems of cocklebur; plants grown at temperature of 75° F. Fig. 10, from untreated plant grown with complete nutrient solution. Fig. 11, from untreated plant grown without calcium; least amount of residue with this treatment; form of residue also different. Fig. 12, from plant grown with complete nutrient solution and treated with alpha-naphthalene acetamide; mineral residue heaviest with this treatment. Fig. 13, from plant grown without calcium and treated with alpha-naphthalene acetamide; mineral residue more similar to that of normal plant than other two treatments.

cium deficiency has not been determined. It may permit greater uptake of calcium from the soil, or possibly it allows more effective re-utilization of calcium by facilitating its conversion from a relatively immobile to a mobile form which may be re-exported from one tissue to another. An available calcium supply would then make possible normal growth of cells. The cell walls in this case would more closely resemble those of normal plants, and proper functioning of the cell membrane would continue for a longer time than if the calcium were permanently fixed where first utilized. It appears possible that studies of this type may be used to determine the relation of growth substances to the mineral nutrition of plants.

Summary

1. Plants of cocklebur were grown with a complete nutrient culture medium and in one lacking calcium. One-half of these plants were treated with alpha-naphthalene acetamide.

2. Symptoms of calcium deficiency, such as short upper internodes, downward cupping of the leaves, brown areas on the surface of the leaves and darkening of the margins, and drying of the stem tips, soon appeared on plants grown without calcium Treatment with alpha-naphthalene acetamide had only a slight effect on delaying symptoms of calcium deficiency at a temperature of 65° F. At 73° F., on the other hand, treatment of the plants with the acetamide greatly delayed the appearance of symptoms of calcium deficiency. At a temperature of 75° F. plants grown without calcium were dry except for a few of the lower leaves after 46 days. Seventy-four days from the start of the treatment plants grown without calcium but treated with the acetamide first began to show slight symptoms of calcium deficiency.

3. The fourth internode of plants grown without calcium had smaller and thinner-walled cells at both 65° F. and 75° F. than the normal plants. When treated with alpha-naphthalene acetamide at 75° F., the cells of calcium-deficient plants more nearly resembled those of the normal plants in size and degree of lignification, but at 65° F. the treatment was less effective; some of the cells had collapsed, giving necrotic-appearing regions. Stems of plants given a complete nutrient and treated with the acetamide had an active cambium and a greater number of xylem elements than the controls.

4. Sections of the stems were incinerated. As compared with control sections those from calcium-deficient plants contained the least ash. Sections from calcium-deficient plants treated with alpha-naphthalene acetamide contained nearly as much ash as the controls and those from the complete nutrient culture plus the acetamide appeared to contain more than the controls.

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THE ENDOSPERM-EMBRYO RELATIONSHIP IN AN AUTONOMOUS APOMICT, TARAXACUM OFFICINALE[1]

D. C. COOPER AND R. A. BRINK

Introduction

In autonomous apomicts the egg and the central cell of the megagametophyte undergo a series of divisions to develop embryo and endosperm, respectively, without fertilization. This is an extreme departure from the behavior typical of the angiosperms. Normally seed development is initiated by double fertilization; one sperm joins with the egg which then gives rise to the embryo; a second sperm nucleus, homologous with the first, conjugates with the central nucleus to form the endosperm mother nucleus. In pseudogamous plants the central cell is fertilized, but the egg develops parthenogenetically. The present study is concerned with the effects of the omission of both fertilization processes on seed development in the common dandelion, *Taraxacum officinale*.

Elsewhere, the writers (1) have summarized the evidence showing that the secondary fertilization characteristic of the angiosperms is basically important for seed development. The endosperm whose development is thus initiated serves two functions: it probably elaborates and certainly accumulates nutrients which the embryo later absorbs; secondly, it exerts a regulatory action on the maternal portions of the seed, particularly during early post-fertilization stages. The megagametophyte, containing the egg and central nucleus, is a small structure imbedded within a relatively massive nucellus and testa. Usually all these tissues are low in food reserves at fertilization. This means that most of the nutrients required for growth of the seed must be moved in from other parts of the plant and then partitioned among embryo, endosperm, and seed coat. The young embryo grows slowly. The maternal tissues, on the other hand, are stimulated to rapid expansion by fertilization. Under these conditions the embryo would appear to be at a disadvantage nutritionally. The endosperm may be pictured as redressing the nutritional balance in favor of the embryo. Usually the endosperm develops rapidly and is assumed to attain quickly a position of physiological dominance in the seed. The secondary fertilization is interpreted as a device which promotes aggressive development of the endosperm. In accordance with this reasoning one would expect that in autonomous apomicts the ends served by the secondary fertilization either would be unnecessary or would be met by another mechanism.

Material and methods

The almost complete fertility of the plant was a principal factor in choosing

[1] Paper from the Department of Genetics, College of Agriculture, University of Wisconsin, no. 403.

the common dandelion as experimental material. In many other apomicts widely varying proportions of the ovules and young seeds are abortive as a result of inherent causes. Their partial fertility is bound up directly or indirectly with the irregular meiotic phenomena characteristic of these forms. GUSTAFSSON's (**2**, **3**) comprehensive review may be referred to for a summary of these relations. Megasporogenesis in the common dandelion is uniform, although atypical, and leads to a full complement of functional ovules. The egg nucleus carries the unreduced number of chromosomes, and fertilization is either absent altogether or occurs so infrequently that the chance of encountering a seed of sexual origin is negligible. Furthermore, since the pollen is nonfunctional as a result of abortion of the microspores and nondehiscence of the anthers, any possible effect of growing pollen tubes on seed development is excluded.

The regular occurrence of autonomous apomixis in *T. officinale* makes impossible a comparison of seed development with a closely comparable sexual form. Another species, *T. kok-saghyz*, the Russian dandelion, therefore, was used as control material. *T. kok-saghyz* is sexual (**5**) and, since it is self-incompatible, requires cross-pollination for seed formation. The species is diploid ($2x = 16$), whereas the common dandelion is triploid ($3x = 24$). Although the difference in ploidy may be significant for apomictic versus sexual reproduction in the two species, it is probably not material to the present investigation.

Biotypes of *T. officinale* occurring as weeds in the vicinity of Madison, Wisconsin, were used. The *T. kok-saghyz* employed was an unpedigreed stock of Russian origin. It was grown in cultivated rows and allowed to open-pollinate.

Parallel series of *T. officinale* and *T. kok-saghyz* heads were collected (*a*) at the late bud stage, (*b*) just prior to anthesis, (*c*) with open flowers, and (*d*) with young fruits ranging from 1 to 6 days of age. A portion of the material of each collection was fixed in Carnoy's solution (3 parts 95% alcohol, 1 part glacial acetic acid, and 1 part chloroform) for 6 hours and then transferred to 70% alcohol. The remainder was fixed in Karpechenko's modification of Nawaschin's fluid for 24 hours and then dehydrated. Since the heads tend to float in an aqueous solution, they were first immersed in Carnoy's solution for 30–40 seconds and then placed in the modified Nawaschin's fluid into which they immediately sank. After both types of fixation the material was dehydrated and imbedded in paraffin, and sections 15–18 μ in thickness were cut, mounted serially, and stained in a number of combinations of stains. Dilute Delafield's haematoxylin followed by safranin and a fast green–safranin combination gave the most satisfactory results.

The developing seeds were examined for number of cells in the embryo and in the associated endosperm. These data were taken on 1118 developing seeds of *T. officinale* and on 310 developing seeds of *T. kok-saghyz* wherein the number of mitotic cycles in the endosperm ranged from none to seven. Observations were made also on the changes occurring in the maternal tissues of the seeds during early stages of development with particular reference to the stainable nutrient materials. Various combinations of stains were used in order to determine the nature of these materials.

MORPHOLOGY OF OVULE

The ovary of *Taraxacum* is unilocular. The ovule arises from the base of the ovarian cavity and, owing to unilateral

growth, assumes an anatropous position early in the course of its development. The nucellus is composed of a single layer of cells which surrounds the megaspore mother cell. It disintegrates during the course of development of the megagametophyte, so that the latter structure comes to lie in direct contact with the innermost layer of cells of the single massive integument. This layer of cells, the endothelium, persists and apparently functions in the transference of nutritive materials during the course of seed development. The mature ovule is an elongate, flattened structure which is oval in shape when seen in cross section. A single vascular bundle enters the ovule through the funiculus and makes an arc about the greatest circumference of the ovule. It terminates in that portion of the integument opposite the funiculus at a level with the inner limits of the micropyle. The megagametophyte is a typical eight-nucleate, seven-celled structure. Little, if any, reserve food material is stored in the megagametophyte.

Results

Seed development in T. kok-saghyz

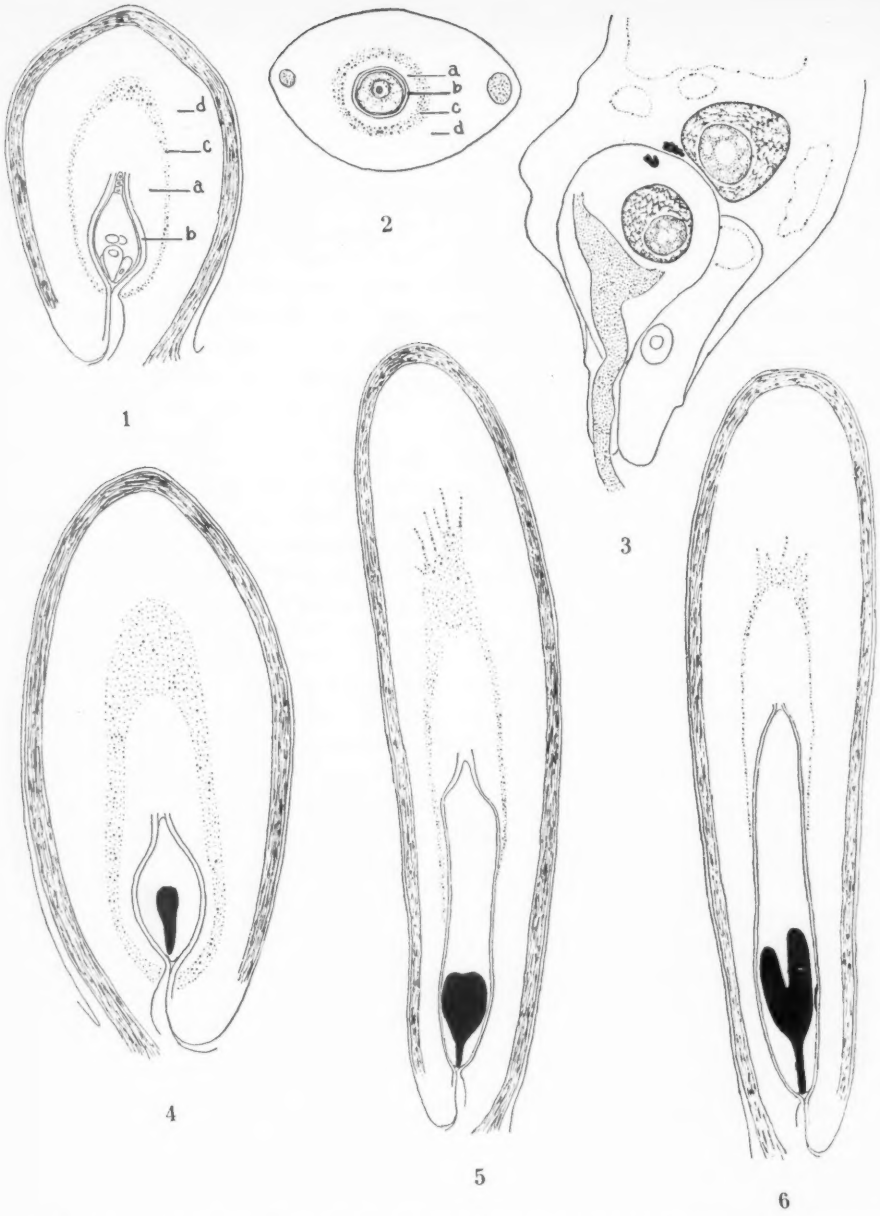
The ovule of *T. kok-saghyz* measures, on the average, 0.66 mm. in length, 0.37 mm. in width, and 0.25 mm. in thickness at the mature megagametophyte stage (figs. 1, 2). The cells of the inner layers of the integument (*a*) adjacent to the endothelium (*b*) lose most of their contents during the expansion and maturation of the gametophyte and contain little more than shrunken and distorted nuclei when the ovule is mature. The cells of the integument (*c*) immediately surrounding this depleted region contain dense cytoplasm and well-defined nuclei. The outermost parenchymatous cells (*d*) are highly vacuolate.

The inflorescence of *T. kok-saghyz* is a terminal head bearing a hundred or more flowers. The buds are at different stages of maturity when the head begins to expand; the peripheral buds are most advanced and open first, so that there are one or more rows of open flowers surrounding a central core of buds; most of the centrally located buds open the following day. Occasionally, on the larger heads, the flowers located in the central region do not open until the second day. All flowers close within a few hours (6–8) after opening.

The buds open early in the morning. Fertilization occurs within a short time after pollination (fig. 3) and stages of gametic union are present by midmorning (8:00–9:00 A.M.). The seed follows the pattern of development characteristic of sexually reproductive angiosperms. Both embryo and endosperm begin growth shortly after double fertilization. Two-celled proembryos associated with two- or four-celled endosperms are present by midafternoon (2:00–3:00 P.M.). The endosperm is cellular throughout the course of its development. No early multinucleate stages, such as described by Warmke (**5**), were found in this material. Both embryo and endosperm continue to grow at a rapid pace, and day-old seeds possess embryos ranging from 6 to 88 cells associated with endosperms ranging from 8 to 128 cells.

The majority of the seeds (70%) have endosperms with 32 or more cells when 1 day old and 64 or more cells some 8 hours later. The number of cells in an embryo associated with a 32-celled endosperm ranges from 11 to 26 (average 16). The embryo continues to grow and ranges from 20 to 52 cells (average 31) in size when associated with a 64-celled endosperm (fig. 4).

The seed enlarges rapidly following fertilization and measures about 0.8 mm. in length and 0.46 mm. in width as seen



FIGS. 1–6.—*T. kok-saghyz*. Fig. 1, median longitudinal section of ovule with mature megagametophyte. Regions of integument containing cells with shrunken nuclei (*a*), endothelium (*b*), cells with dense cytoplasm and plump nuclei (*c*), and highly vacuolate cells (*d*) are shown. ×78. Fig. 2, transverse section of mature ovule showing regions as described in fig. 1. ×78. Fig. 3, micropylar portion of megagametophyte showing double fertilization. ×700. Fig. 4, median longitudinal section of day-old seed. ×78. Fig. 5, 2-day-old seed. Embryo with cotyledonary initials. ×39. Fig. 6, 3-day-old seed. Embryo with elongating cotyledons. ×39.

in median longitudinal section by the time the zygote and endosperm mother cell have completed their first divisions. This enlargement continues, and there is a close correlation between growth of endosperm, as indicated by cell number, and increase in size of seed during the early stages of seed development (table 1). Only those seeds for which a median longitudinal section was available for measurement were recorded. The young seed elongates with little or no increase in width during its initial stages of development. Thereafter it slowly expands in width and increases greatly in length.

Heads collected 32 hours after the opening of the peripheral buds possess fruits at different stages of development. The seeds in the majority of the fruits about the periphery of the inflorescence have embryos associated with endosperms consisting of 64 or more cells. The more centrally located fruits have seeds with 2-celled proembryos and 2- to 4-celled endosperms.

Active mitosis continues in the embryo so that a spherical mass of cells is formed at the tip of the suspensor. The embryo gradually elongates and broadens at the apex. Two groups of cells on opposite sides of the periphery of the apex as well as those in the basal region become actively meristematic, initiating the development of the cotyledons and hypocotyl (fig. 5).

The 2-day-old seeds possess embryos ranging from a spherical mass of cells to structures with cotyledonary initials and an elongating hypocotyl. The number of cells in the spherical embryos varies from 140 to 216. Such embryos are associated with endosperms consisting of 256 cells. Following the initiation of cotyledon and hypocotyl development the embryo grows more rapidly than, as well as at the expense of, the endosperm (fig. 6).

The inner layers of cells of the integument, which are partially broken down at the time of fertilization, become more or less completely digested during the early stages of seed development. As the endosperm expands, the endothelium comes into direct contact with the intermediate layers of densely cytoplasmic cells. The latter cells appear to act as an absorbing tissue in the region opposite the chalazal end of the endosperm. Nutritive materials are undoubtedly transferred from this tissue to the chalazal end of the endosperm and through the endothelium with which it is directly associated laterally to the endosperm.

TABLE 1

GROWTH OF SEEDS OF T. KOK-SAGHYZ

Endosperm	Embryo	Average size median longitudinal sections of seed (mm.)
2 cells	2 cells	0.8 ×0.46
32 cells	26 cells	0.87×0.47
64 cells	52 cells	0.9 ×0.46
128 cells	62 cells	0.95×0.47
256 cells	220 cells	1.4 ×0.55
Multicellular	Flattened at apex	1.66×0.59
Multicellular	Elongating cotyledons	2.35×0.58

As the seed continues to grow, there is active mitosis in the chalazal end of the endosperm and rapid digestion of this tissue in the region of the embryo. The endosperm continues to act as the nurse tissue for the embryo which develops rapidly at its expense. Little, if any, endosperm remains in the mature seed. Maturation of the seed is completed between 9 and 12 days after fertilization.

SEED DEVELOPMENT IN T. OFFICINALE

The ovule of *T. officinale* is shorter than that of *T. kok-saghyz* at the mature

megagametophyte stage (fig. 7). It measures 0.43 mm. in length, on the average, but its width and thickness approximate those of the ovule of the latter species (fig. 8). The cells of the integument (*a*) just outside the endothelium (*b*) increase in size as the ovule matures. These cells act as a storehouse for a homogeneous material which, by its staining reaction, appears to be proteinaceous in nature. It takes a brilliant red color when stained with safranin and a deep blue with Delafield's haematoxylin. It also stains heavily with Orange G, eosin, and picric acid.

TABLE 2

GROWTH OF SEEDS OF T. OFFICINALE

Endosperm	Embryo	Average size median longitudinal sections of seed (mm.)
1 cell	13 cells	0.54×0.36
2 cells	18 cells	0.53×0.39
4 cells	11 cells	0.54×0.37
16 cells	21 cells	0.54×0.38
64 cells	73 cells	0.82×0.49
128 cells	105 cells	0.93×0.50
Multicellular	Flattened at apex	2.13×0.70
Multicellular	Elongating cotyledons	2.78×0.68

The inflorescence of *T. officinale* is a terminal head bearing many flowers. All florets are at approximately the same stage of development when the head begins to expand. The peripheral florets open early in the morning, and gradually the more centrally located ones unfold, so that all have opened by midday. The flowers close toward night. The head does not expand again the next day.

Active development of the seed is initiated in the late bud stage. The embryo usually starts to develop in advance of the endosperm (fig. 9). Ovaries collected 1 day prior to the opening of the flowers possess seeds wherein embryos, consisting of one to 30 or more cells, are associated with 1-, 2-, and 4-celled endosperms. Growth of the seed is rapid, and various stages of development are present at the open-flower stage (figs. 10, 12). An examination of 207 seeds collected when the flowers were beginning to open revealed the presence of embryos ranging in size from 1 to 108 cells associated with endosperms in varying stages of growth (1 to 159 cells.)

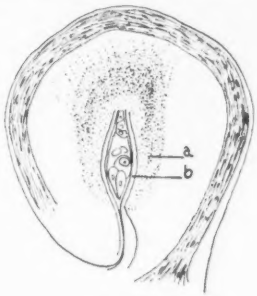
There is no correlation between the stage of seed development and the relative position of the fruit in the head. Fruits containing seeds in the more advanced stages of growth are scattered at random. Even the stages of development found in seeds of adjacent fruits differ widely. The seeds of the peripheral fruits are no more advanced than those of the centrally located fruits.

The seed enlarges slowly with the onset of embryo and endosperm formation. Measurements of median longitudinal sections of 21 seeds containing endosperms ranging in cell number from 1 to 16 showed little variation in size (table 2). These seeds, on the average, had a length of 0.54 mm. and a width of 0.38 mm. None of them had reached the size of the mature ovule of *T. koksaghyz*. The seeds expand rapidly thereafter so that seeds of the two species with 128-celled endosperms are comparable in size.

The seeds of *T. officinale* continue to grow more rapidly than those of *T. koksaghyz*. This is due to a greater increase in size of the endosperm and integument in the former species. Measurements of seeds containing embryos flattened at the apex (fig. 13) or with elongating cotyledons (fig. 14) show that those of *T. officinale* are distinctly larger than those of the related sexual species.

The innermost layers of cells of the in-

tegument adjacent to the endothelium become greatly enlarged and highly vacuolate during the period in which maturation of the megagametophyte occurs. A sheath of cells with dense cytoplasm immediately surrounds this highly vacuolate tissue. The chalazal portion of the sheath is five to six cell layers in thickness. It gradually tapers down laterally, so that it is one or two layers thick at the level of the egg apparatus. The outermost layers of integumentary cells, that is, those between the sheath and the vascular tissue or epidermis, form a vacuo-



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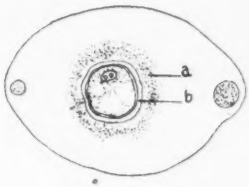

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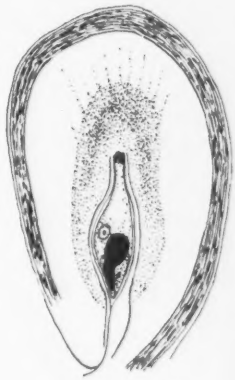

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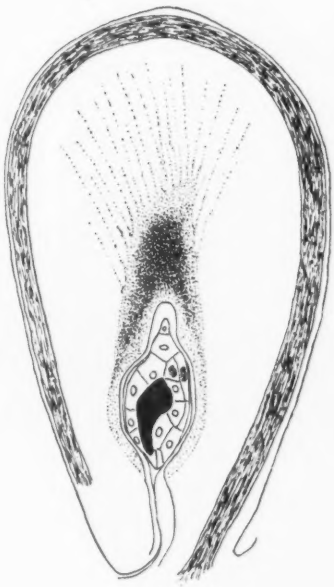

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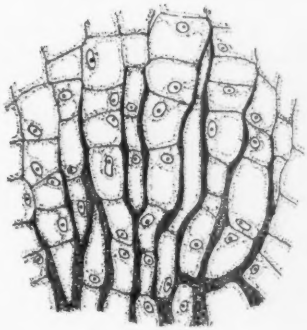

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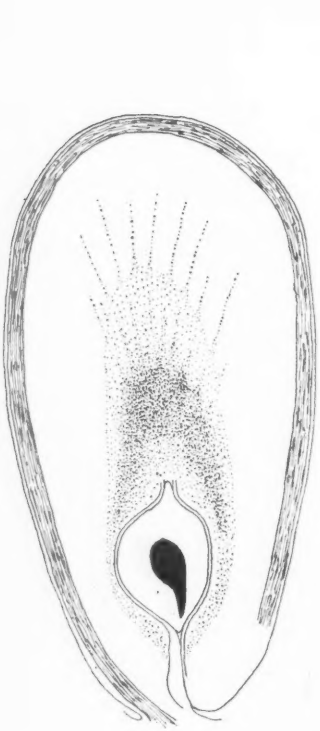
FIGS. 7–11.—*T. officinale*. Fig. 7, median longitudinal section of ovule with mature megagametophyte. (*a*) Storage region of integument, (*b*) endothelium. ×78. Fig. 8, transverse section of ovule showing storage region of integument. ×78. Fig. 9, seed at late bud stage with developing embryo and one-celled endosperm. ×78. Fig. 10, seed at open-flower stage with developing embryo (51 cells) and endosperm (32 cells). ×78. Fig. 11, portion of integument at outer edge of storage region showing viscous material between cells. ×350.

late parenchymatous tissue with little, if any, storage materials.

The intermediate sheath of cells with dense cytoplasm becomes differentiated as an absorbing and secretory tissue just prior to the onset of seed development. A viscous material collects between the cells and passes into the underlying region of highly vacuolate cells where it is stored (fig. 11). As the endosperm expands during seed development these storage materials are gradually utilized as nutrients.

Strands of elongate cells with dense cytoplasm become differentiated between the secretory tissue of the integu-



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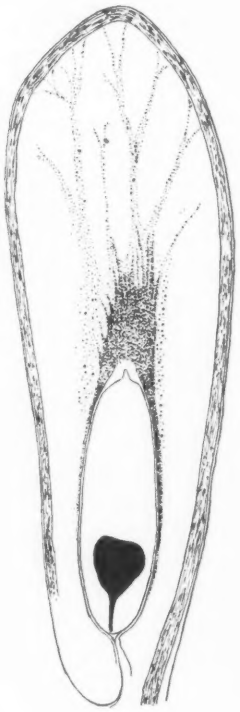

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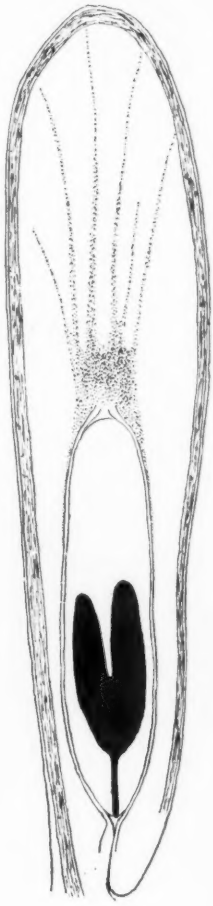

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FIGS. 12–14.—*T. officinale*. Fig. 12, seed at open-flower stage with 105-celled embryo and 128-celled endosperm. ×78. Fig. 13, seed 1 day after open-flower stage. Embryo flattened at apex. Elongated strands of conducting cells extend from storage region of integument to vascular bundle. ×39. Fig. 14, seed 2 days after open-flower stage. Embryo with elongating cotyledons. ×39.

ment and the vascular bundle forming a pathway for the movement of materials from the vascular tissue to the storage area in the integument (figs. 13, 14). The parenchymatous cells other than these strands become progressively enlarged and break down during growth of the seed, so that the integument is filled with large empty spaces.

Endosperm-embryo relations

The data on growth of endosperm and embryo in *T. officinale* and *T. kok-saghyz* seeds are summarized in tables 3 and 4. Since cell number in the endosperm increases geometrically, growth of the tissue may be accurately expressed in terms of division cycles. The zero point corresponds to the single mother cell. Cell number doubles thereafter at each successive division. Embryo cell number, on the other hand, varies continuously, so that any grouping of the values into classes is purely arbitrary. A logarithmic scale has been used in table 4 as a convenient way of dealing with the extraordinary variability in size of the *T. officinale* embryos. The class values are the geometric means of the respective classes. The upper limit of each class is thus equal to the class value times the square root of two.

Average growth

Growth of the *T. kok-saghyz* embryo relative to that of the associated endosperm follows the pattern characteristic of the angiosperms (table 3). Following simultaneous initiation by double fertilization, the two tissues develop in tune with each other. Although it grows more rapidly than the embryo, the endosperm is less precocious than in many other sexual species (1). Endosperm cell number is only about twice the embryo cell number in the juvenile *T. kok-saghyz* seed. Perhaps the more significant relation to be deduced from these facts is that, comparatively speaking, early growth of the *T. kok-saghyz* embryo proceeds normally even though the endosperm develops slowly.

The average stage of advancement of the young *T. officinale* embryo in terms of size of the associated endosperm is grossly different from that in the sexual species. Previous to division of the endo-

TABLE 3

MEANS AND STANDARD DEVIATIONS OF EMBRYO CELL NUMBERS DISTRIBUTED ACCORDING TO ENDOSPERM DIVISION CYCLE

	Species	Endosperm division cycle							
		0	1	2	3	4	5	6	7
Number of seeds examined	*T. officinale*	227	253	145	108	111	115	99	60
	T. kok-saghyz	.....	77	32	25	34	68	55	19
Mean embryo cell number	*T. officinale*	15.5	14.5	18.1	21.6	27.2	34.4	56.2	91.5
	T. kok-saghyz	1.0	1.6	2.0	4.4	7.6	13.2	30.0	68.0
Standard deviation of embryo cell number	*T. officinale*	15.6	13.6	17.0	21.1	19.2	24.2	29.9	51.0
	T. kok-saghyz	0	0.5	0.2	0.7	1.9	4.1	9.0	16.7
	F values	.....	773*	9629*	891*	105*	3407*	11.1*	9.3*
Coefficient of variability	*T. officinale*	100.0	93.8	93.9	97.6	70.6	70.3	53.2	55.7
	T. kok-saghyz	.....	31.2	10.0	15.9	25.0	31.0	30.0	26.4

* Significant at 1% level.

sperm mother cell the mean embryo size is about 15 cells. That is to say, considerable early embryo development occurs in the apomict before growth of the endosperm begins. The subsequent increase in size of the *T. officinale* embryo on the average, rather closely parallels that in *T. kok-saghyz* (table 3, fig. 15). At each of the seven endosperm stages represented the embryos of parthenogenetic origin contain 15–20 more cells than the sexual ones. Both classes of embryos show marked acceleration in growth when the endosperms reach the fifth nuclear division cycle.

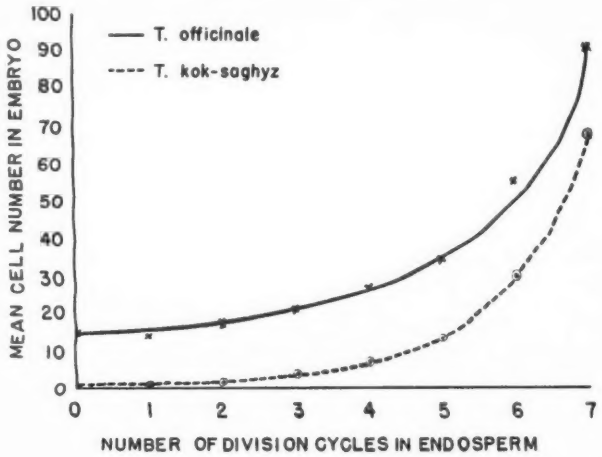

FIG. 15.—Early growth of embryo of *T. kok-saghyz* and *T. officinale* in relation to endosperm size

VARIABILITY IN GROWTH

The mean values for embryo size (table 3) have little significance for comparative purposes unless considered in connection with the associated variability. Reference to table 4 shows that the distributions from which the respective series of *T. officinale* and *T. kok-saghyz* means were computed differ greatly. At the endosperm-mother-cell stage (0-cycle), for example, entries for *T. officinale* occur in all the classes for embryo cell number from 1 to 128. At this stage of endosperm development in *T. kok-saghyz*, on the other hand, all the embryos observed were one-celled. The mode shifts slowly to the right in the next four *T. officinale* distributions but the entries are still spread over all the embryo size classes. In contrast, the embryo cell numbers for *T. kok-saghyz* in each frequency distribution all lie in two, or three, contiguous groups. Similar differences in variability characterize the two kinds of seeds at the more advanced stages observed.

The data on variability in embryo size within each of the eight endosperm classes are summarized in table 3 in terms of the standard deviation and the coefficient of variability. Both these measures reveal a much higher order of dispersion for *T. officinale* than for *T. kok-saghyz* embryos at all the stages of endosperm development represented. The difference in absolute variability, as measured by the standard deviation, is tenfold in the seed class in which four cycles of nuclear

division in the endosperm have occurred. The extent to which the absolute variability in embryo size in *T. officinale* exceeds that in *T. kok-saghyz* throughout the series is illustrated graphically in figure 16. The differences in the standard deviations are highly significant as shown by the *F* values in table 3. The excessive variation in embryo size for a given size of endosperm in the common dandelion obviously constitutes an important feature of seed development in this species.

TABLE 4

DISTRIBUTION OF EMBRYOS BY CELL NUMBER RELATIVE TO ENDOSPERM DIVISION CYCLE

Endosperm division cycle	Species	Total seeds examined	Embryo cell number—logarithmic class values									Standard deviation
			1	2	4	8	16	32	64	128	256	
0	*T. officinale*	227	9	16	33	57	66	38	7	1	.....	15.6
	T. kok-saghyz	All	All	.....	.....	.....	.....	.....	.....	.....	.....	0
1	*T. officinale*	253	23	11	37	70	55	50	6	1	.....	13.6
	T. kok-saghyz	77	31	46	.....	.....	.....	.....	.....	.....	.....	0.5
2	*T. officinale*	145	18	11	7	23	43	33	9	1	.....	17.0
	T. kok-saghyz	32	1	31	.....	.....	.....	.....	.....	.....	.....	0.2
3	*T. officinale*	108	12	6	6	19	27	25	12	1	.....	21.1
	T. kok-saghyz	25	.....	.....	22	3	.....	.....	.....	.....	.....	0.7
4	*T. officinale*	111	4	1	2	9	39	40	14	2	.....	19.2
	T. kok-saghyz	34	.....	.....	5	27	2	.....	.....	.....	.....	1.9
5	*T. officinale*	115	6	.....	4	4	23	50	23	5	.....	24.2
	T. kok-saghyz	68	.....	.....	.....	24	40	4	.....	.....	.....	4.1
6	*T. officinale*	90	1	1	.....	.....	7	31	46	13	.....	29.9
	T. kok-saghyz	55	.....	.....	.....	.....	10	41	4	.....	.....	9.0
7	*T. officinale*	60	2	.....	1	.....	1	8	17	28	3	51.0
	T. kok-saghyz	19	.....	.....	.....	.....	.....	3	16	.....	.....	16.7

The coefficient of correlation between number of cells in endosperm and embryo is $r = 0.57$ for *T. officinale* and $r = 0.76$ for *T. kok-saghyz*. These values are based upon 1118 and 310 seeds, respectively. The regression is nonlinear in both cases, so that r is only an approximate measure of the covariability. It is evident from these values, however, that there is a much closer average relationship between endosperm and embryo size in the sexual than in the apomictic species. But, if the correlation coefficient of 0.57 for the common dandelion discloses the general trend in this species, it also fails to reveal much of significance for an understanding of the actual biological relationship between endosperm and embryo in the apomict. An examination of the conditions in individual seeds is necessary for a full appreciation of the facts.

The embryo of *T. kok-saghyz* is invariably at the zygote stage during the period when the endosperm is a unicellular structure. Slightly more than half the embryos have advanced to the two-celled condition by the time the endosperm is two-celled. Nearly all the embryos are two-celled by the time two mitotic divisions in the endosperm have

occurred, and none exceed this size. Conditions stand in marked contrast in the young *T. officinale* seed. Accompanying unicellular endosperms, embryos were observed varying in size from 1 to 112 cells. Eight seeds among 227 in this class contained embryos with 50 or more cells. Among the 253 seeds observed in which one mitotic division in the endosperm had taken place, 5 contained embryos ranging in size from 52 to 102 cells. One hundred and eight seeds were examined in which the endosperm cells had passed through three division cycles. Six of these seeds contained embryos with more than 50 cells, ranging from 53 to 93.

It should be emphasized that these common dandelion seeds with retarded endosperms but relatively far advanced embryos appeared to be growing just as vigorously at the time of collection as those with much smaller embryos. No evidence was observed which would indicate that they were less likely to attain normal maturity than seeds with any other size ratio of endosperm and embryo.

No less remarkable is the fact that the embryo may remain in the one-celled stage even though the accompanying endosperm passes through several cycles of cell division. Seventy-five *T. officinale* seeds in all were examined in which the egg had not divided. In two of these the endosperm contained 128 cells, in one other there were 64 cells, and in six of the seeds, 32 endosperm cells were present. The eggs in all cases appeared to have remained capable of division. These extreme examples make it clear that in the apomictic species growth of endosperm and embryo are independent of each other to a very high degree.

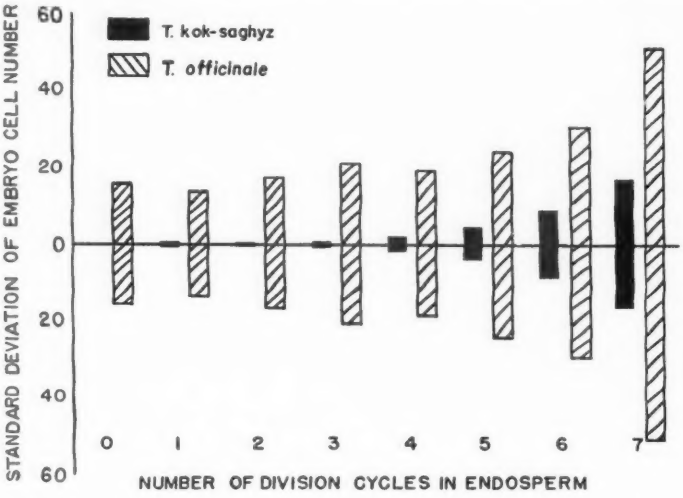

FIG. 16.—Relative variability of embryo size in *T. kok-saghyz* and *T. officinale* at successive stages of endosperm development.

Discussion

Apomixis involves a succession of changes in the reproductive behavior of the plant. The attention of histologists has been directed largely to the meiotic innovations associated with the phenome-

non. The variety and manifest significance of these processes have tended to overshadow other events equally important for completion of the generative cycle. As a result, parthenogenesis in the egg and the conditions promoting development of the embryo have remained largely unexplored. In view of what has been learned in sexual species concerning the basic role of the endosperm in seed formation a study of this tissue in an autonomous apomict promised to be instructive. Since the course of seed development at the early stages largely determines the subsequent behavior, and this varies in species in which abortion occurs later, a fully seed-fertile apomict was sought for the investigation. The common dandelion eminently fulfils this requirement.

Others (4) have recognized that dependence of the young embryo upon the endosperm appears to be relaxed in apomicts. It was desired in the present study to measure the extent to which this is true and to determine whether a compensating mechanism is operative in seed development.

The endosperm-embryo relation in *T. kok-saghyz*, a sexual species studied as a control, conforms in general to the usual pattern in flowering plants. Both tissues originate as fertilization products and subsequently develop in tune with each other. The endosperm, however, is less precocious than usual. Possibly this fact is significant for the occurrence of apomixis in some members of this genus. The writers (1) have pictured the endosperm as performing in the very young seed certain processes which are essential to embryo growth and which the embryo is incapable of performing and can effect by itself only at a more advanced stage of differentiation. Perhaps the *Taraxacum* embryo becomes able to take over these functions from the endosperm very early, thus lowering the barrier to apomictic seed development which the normal endosperm-embryo relationship may be assumed to represent. Evidence on this point from other genera in which apomixis occurs would be of interest. Aside from this restricted early endosperm growth the *T. kok-saghyz* seed appears essentially like that of other sexual angiosperms. Little reserve food material is present in the ovule at fertilization, and it is probable that the young endosperm plays its usual role of absorbing nutrients moved in from other parts of the plant and conveying them in suitable form to the embryo.

A radically different set of conditions is presented in the apomictic *T. officinale* seed. Omission of fertilization of both the egg and the central cell of the megagametophyte means that development of embryo and endosperm may start independently. This, in fact, is what happens. Furthermore, it appears to be purely a matter of chance which divides first, the egg or the endosperm mother cell. There is no indication that the initiation of division in the one influences that of the other.

Endosperm and embryo in the apomict are independent of each other to a considerable degree in their further development. The correlation coefficient (r) between cell number in the embryo and in the endosperm for all 1118 *T. officinale* seeds examined proved to be 0.57. In spite of the positive statistical relationship between the two variables which this value indicates, the extent to which growth of the embryo is actually dependent upon a developing endosperm appears to be low. This conclusion is supported by the fact that apparently healthy seeds with unicellular endosperms were found whose embryos never-

theless had advanced as far as the 112-celled stage. Such cases show that either the unicellular structure is extraordinarily efficient in performing the functions of an endosperm or the embryo of the common dandelion is largely able to dispense with this tissue. Similarly it may be argued that a well-developed endosperm is not necessarily an advantage to the associated embryo, since seeds occur with one-celled embryos accompanied by endosperms containing as many as 256 cells. These extreme examples of disparity in stage of development between endosperm and embryo in healthy seeds indicate that the functional relationship between the two tissues is low.

How, then, is nourishment of the apomictic *T. officinale* embryo accomplished if not through the mediation of an endosperm as in sexual species? The answer to this question appears to lie in the rich supply of nutritive material stored in the *T. officinale* ovule. The endosperm is rendered superfluous through completion of its principal function by another tissue even before the egg divides. The preanthesis development of many embryos can be explained on this hypothesis, and it accounts also for the ability of the embryo to develop to advanced stages in the absence of an active endosperm. The embryo draws directly upon a food supply already present. The nutrients required for embryo growth are presynthesized in the ovule and stored in the integument immediately adjacent to the gametophyte. Hence transformation of the ovule into a seed in the apomict does not wait upon generation of an endosperm as in the sexual form. The essential step in this process, namely, provision for nourishment of the embryo, is consumated in the pre-seed stage.

Summary

1. A study was made of the endosperm-embryo relationship in the developing seed of *Taraxacum officinale*, a fully fertile, autonomous apomict. A related sexual species, *T. kok-saghyz*, served as the control.

2. Seed development in *T. kok-saghyz* follows the pattern characteristic of the angiosperms. Endosperm and embryo development are initiated by double fertilization, and the two tissues are closely correlated in their subsequent growth. The endosperm is less precocious, however, than in most flowering plants. The mature ovule is low in food reserves.

3. The *T. officinale* ovule stores a large mass of protein-rich nutrient material in the integument. Endosperm and embryo begin development without fertilization and apparently quite independently of each other. During the ensuing growth there is extreme diversity in the size ratios of the two tissues from seed to seed in the same head. For example, the embryo may attain a relatively advanced, and normal, development, while the endosperm remains one-celled. Conversely, the endosperm may become multicellular prior to division of the egg.

4. Explanation of the fact that in the apomict embryo development may proceed normally in spite of even very limited endosperm growth probably lies in the extensive prestorage of food materials in the *T. officinale* ovule. The basic nutritive function which the endosperm performs in most flowering plants is thus rendered superfluous in this case.

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CARBOHYDRATE FRACTIONS OF GRAPEFRUIT PEEL[1]

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Introduction

The dry matter of grapefruit peel is composed chiefly of carbohydrates, and the physical and chemical properties of the peel are related to its carbohydrate content. For example, the physiological breakdown of the fruit while on the tree and in storage, and the deleterious effects caused by the invasion of fungi, result in chemical changes in the carbohydrate constituents of the peel. Citrus peels, in general, exhibit hydrophilic properties, and the degree of hydration is dependent not only upon the total ionic concentration but also upon the amount of certain carbohydrate fractions contained in the peel. To understand the biochemical mechanisms occurring in grapefruit peel, it is necessary to know its composition and the relation of one constituent to another. Such data are also essential for the understanding of the physiological relations between the peel and the pulp and for the understanding of the metabolic reactions of the peel under storage conditions.

The present studies were concerned with the carbohydrate fractions of grapefruit peel. Samples of peel were thoroughly extracted with 80% ethyl alcohol and separated into the alcohol-soluble and alcohol-insoluble fractions. The alcohol-soluble fraction contains chiefly the mono- and disaccharides; it also contains other substances, such as essential oils and waxes and various concentrations of undetermined substances. The alcohol-insoluble fraction contains the cell-wall constituents, such as cellulose, lignin, pectin, and hemicellulose (sometimes designated as polysaccharides or acid-hydrolyzable material). These substances appear to be closely related to one another, especially pectin and hemicellulose.

The pectins are not usually considered part of the carbohydrate reserve of the plant. The readily available carbohydrate fractions that serve as sources of energy consist of the total soluble sugars and the starch-dextrin fraction. The carbohydrate material of the cell walls is not necessarily composed of condensation products of the simple sugars but may consist of uronic acids united with the hexose and pentose sugars. The pectins belong to this general class of compounds, and any hemicellulose fraction that may accompany the pectins on weak-acid extraction of the tissues probably contains a uronic acid group. Cellulose, hemicellulose, and lignin compose the structural, and not the energy-yielding, units of the tissues; consequently, they are relatively inert to the metabolic activity of the cells.

[1] Paper no. 611, University of California Citrus Experiment Station, Riverside, California.

Material and methods

Alcohol-insoluble solids and alcohol-soluble solids were determined on the peel from samples of fifty to one hundred mature grapefruits each. Fruit samples were taken from trees that had been either fumigated with hydrocyanic acid (HCN) or sprayed with oil during the growing season. Since these are usual orchard practices, it was desirable to determine any differences in carbohydrate composition of the peel that might exist between the two treatments.

For moisture determinations, disks of the peel were removed from the equatorial region of the fruit with a cork borer (no. 9). Other disks taken from this and other areas of the peel were weighed and used for quantitative determination of the alcohol-insoluble and alcohol-soluble solids. A much larger sample of the remaining peel was used for obtaining a large experimental sample of alcohol-insoluble material.

For quantitative determinations on the small-disk samples, the fresh peel was finely ground to a fluid state in a Waring blendor in the presence of 95% ethyl alcohol. These samples were immediately heated to boiling temperature and allowed to stand overnight in 300 ml. of 80% ethyl alcohol, a correction being made for the moisture in the sample. Two additional extractions were made with hot 80% ethyl alcohol; for each extraction the samples remained in the extractant for 24 hours. The insoluble material was filtered off and washed thoroughly, first with 95% ethyl alcohol and finally with petroleum ether. The colorless product was dried to constant weight in an oven at 65° C. and weighed quantitatively. The percentages of alcohol-insoluble solids were calculated from this weight.

Pectin was extracted from the alcohol-insoluble solids by placing 0.5 gm. of the material in a 150-ml. beaker and adding 50 ml. of hot water. The beaker was placed on an asbestos gauze over a low flame, and the mixture was gently heated to slow boiling for a 10-minute extraction period. While hot, the extract was filtered with suction through a hardened filter paper (11-cm., Whatman no. 50). The solid material was scraped from the paper and returned to the beaker with 50 ml. of boiling water, and the process was repeated. A third extraction was made in the same way, all the filtrates being received in the same flask. The combined solution was filtered by suction through a small, soft filter paper (Whatman no. 42), and the filter was washed with hot water. The extract was transferred to a 600-ml. beaker and diluted to 300-ml. volume.

Pectin in the aqueous extract was determined as calcium pectate by the method of CARRÉ and HAYNES (4), with slight modifications. The pectin was saponified by adding 6 ml. of 5 *N* NaOH to the 300-ml. aqueous extract, and the solution was allowed to stand for 1 hour. Fifty milliliters of *N* acetic acid were added, with stirring, and, after 5 minutes, 50 ml. of *M* calcium chloride were added and stirred. The solution was allowed to stand 1 hour or longer for the complete precipitation of calcium pectate. The precipitate was filtered by suction through a hardened filter paper (11-cm., Whatman no. 50) on a Büchner funnel. When the volume in the funnel had been reduced to about 50–75 ml., the precipitate was washed with 200–250 ml. of boiling water. The washing was continued until the filtrate was free of chlorides. (The volume of material in the funnel must not be less than 50 ml. at any time; otherwise, filtering becomes too slow and washing difficult.) When washing was

complete, the precipitate was sucked dry enough so that it would adhere to the paper when it was lifted from the funnel. The matched paper was then wetted with distilled water, and both papers were placed in an oven and dried to constant weight at 105° C., using the balanced paper as tare. The dried calcium pectate loosens from the paper and is readily removed as a thin film for analysis.

The material from the alcohol-insoluble solids remaining after three extractions with boiling water was then extracted in a similar manner with three successive 50-ml. portions of hot $N/75$ HCl (pH 2.5). The combined solutions were filtered through soft paper and diluted to 300 ml. Sufficient concentrated HCl was added to the extract, with stirring, to make a 10% solution. This precipitated pectinic acid in a form which could be filtered readily, with suction, through a hardened filter paper. Without washing, the paper and precipitate were returned to the beaker. About 100 ml. of hot water were added, together with one drop of phenolphthalein solution and enough NH_4OH to produce a faint pink. The pectinic acid readily dissolved. The paper was returned to the funnel, and the solution was drawn through it two or three times to dissolve any precipitate adhering to the funnel. Finally, it was washed with hot water. The total volume was again about 300 ml. Calcium pectate was precipitated and treated as in the aqueous extract, except that it was filtered onto a 7.0-cm. paper.

The residue remaining after the HCl extraction was washed with hot water, transferred to a weighed watch glass, and dried at 105° C. This residue was designated as the cellulose and hemicellulose fraction (**21**).

Alcoholic extracts and washings were combined and freed of alcohol by evaporation on a steam bath. The residue was extracted with warm water to dissolve the soluble sugars. The aqueous extract was diluted to a definite volume, and aliquot portions were taken for analysis.

Sugar determinations were made by the HAGEDORN and JENSEN (**7, 8**) method as modified by BLISH (**1, 2**). The strength of the reagents employed by BLISH was satisfactory for determining the reducing and total sugars as glucose when the values ranged from 3 to 10 mg. in 10 ml. of peel extract. When necessary, the samples were diluted so that the values fell within this range. This method was used because comparative tests showed that it was rapid and that it gave comparable results for the quantity of sugar in the sample. The glucose factor of the reagents was determined with a sample furnished by the National Bureau of Standards.

Uronic acids of the samples were estimated by determining CO_2 by the method of LEFEVRE and TOLLENS (**13**), as modified by Dickson *et al.* (**5**).

Methoxyl groups were determined by the saponification procedure of ROMEO (**18**). Values obtained by this method compare favorably with those obtained by the ZEISEL method (**22, 23**).

Furfural determinations were made by the colorimetric procedure described by BRYANT *et al.* (**3**).

Calcium was determined volumetrically by treating the oxalate with dilute H_2SO_4 and subsequently titrating the liberated oxalic acid with standard $KMnO_4$.

Results

ALCOHOL-SOLUBLE FRACTION

In the peel of fumigated and oil-sprayed fruit the dry matter soluble in 80% ethyl alcohol averaged 66.31% and

67.35%, respectively (table 1). These results are higher than those reported for lemon peel (21). They are approximately equivalent to values determined by HARVEY and RYGG (10), who found that the soluble solids and total sugars in grapefruit peel increased steadily through the season and that the total sugars accounted for slightly more than half of the soluble solids.

considerably greater than that of sucrose. Fluctuations in the concentration of these peel constituents are markedly influenced by the maturity of the fruit. In the alcoholic extract from both sets of samples approximately 40% of the solids were in a form other than reducing sugars and sucrose. The alcohol-soluble fraction contained substances which liberated a small amount of CO_2 (mean, 0.67% for fumigated samples and 0.63% for oil-sprayed samples) on hydrolysis with 12% HCl.

TABLE 1

ANALYSIS OF ALCOHOL-INSOLUBLE AND ALCOHOL-SOLUBLE SOLIDS OF GRAPEFRUIT PEEL

Sample no.	Moisture (% fresh weight)	Alcohol-insoluble solids (% dry weight)	Alcohol-soluble solids (% dry weight)	CO_2 produced on hydrolysis with 12% HCl: Alcohol-insoluble solids		Alcohol-soluble solids		% Methoxyl in A.I.S.*	Ratio, methoxyl to CO_2 in A.I.S.	Water-soluble sugars (as glucose) in alcoholic extract (% dry weight)			Total sugars (% A.S.S.)
				% dry weight*	% A.I.S.*	% dry weight	% A.S.S.			Reducing	Sucrose	Total	
	Fumigated fruit												
1	81.46	33.47	66.53	3.51	10.48	0.42	0.63	5.71	0.545	24.12	15.89	40.01	60.14
2	81.33	33.42	66.58	3.46	10.35	.45	.68	5.66	.547	24.08	15.25	39.33	59.07
3	81.52	33.69	66.31	3.53	10.48	.44	.66	5.73	.547	23.97	16.68	40.65	61.30
4	81.40	33.84	66.16	3.54	10.47	.42	.63	5.78	.552	24.19	16.04	40.23	60.81
5	81.46	34.04	65.96	3.55	10.43	.49	.74	5.71	.547	25.38	15.84	41.22	62.49
Mean	81.43	33.69	66.31	3.52	10.44	0.44	0.67	5.72	0.548	24.35	15.94	40.29	60.76
	Oil-sprayed fruit												
1	82.23	32.95	67.05	3.45	10.48	0.49	0.73	5.71	0.545	24.87	17.46	42.33	63.13
2	82.01	32.65	67.35	3.37	10.32	.39	.58	5.66	.548	24.53	15.57	40.10	59.55
3	82.13	32.73	67.27	3.41	10.42	.51	.76	5.75	.552	25.98	16.34	42.32	62.90
4	82.11	32.26	67.74	3.34	10.37	.39	.58	5.78	.557	25.31	16.24	41.55	61.34
5	82.23	32.65	67.35	3.37	10.32	.33	.49	5.66	.548	24.91	15.17	40.08	59.50
Mean	82.14	32.65	67.35	3.39	10.38	0.42	0.63	5.71	0.550	25.12	16.16	41.28	61.28

* Calculated on ash-free, moisture-free basis.

In the present experiment the total sugars accounted for 60.76% and 61.28%, respectively (mean values), of the alcohol-soluble solids in the peel of the fumigated and oil-sprayed fruit. On a dry-weight basis, the total sugars averaged 40.29% and 41.28%, respectively. The amount of reducing sugars was con-

ALCOHOL-INSOLUBLE FRACTION

The alcohol-insoluble solids, which are composed chiefly of the structural constituents of the cells (cellulose, hemicel-

lulose, pectin, protein, etc.), amounted to 33.69% and 32.65% of the dry weight of the peel of fumigated and oil-sprayed fruit, respectively. This fraction is well suited for a study of these structural constituents, as it is free of all soluble carbohydrates and pigments and of a considerable portion of the ash.

The efficiency with which alcohol separates the pectins of grapefruit peel is shown in table 1. The CO_2 produced by the peel on hydrolysis with 12% HCl is quantitatively related to the amount of uronic acid in the sample (**13**). The uronic acids yield 22.50% CO_2. Since pectin is chiefly composed of uronic acids, the yield of CO_2 by the fractions should give a fair approximation of the pectin content. Pectin is relatively insoluble in alcohol; therefore, most of the CO_2 should be liberated in the alcohol-insoluble-solids fraction. This fraction yielded, on the average, 10.44% and 10.38% of its weight as CO_2, in fumigated and oil-sprayed fruit, respectively. These values are significantly higher than those reported for lemon peel (**21**).

The high percentage of CO_2 in the alcohol-insoluble solids results from the fact that a large proportion of the dry matter is soluble in 80% alcohol. Since 80% alcohol does not remove pectin from the peel, it follows that, the lower the percentage of alcohol-insoluble solids in the dry matter, the higher the percentage of CO_2 in this fraction. On the assumption that all the CO_2 liberated from the alcohol-insoluble fraction is derived from the pectin, the percentage of pectin in this fraction can be estimated by the simple calculation $10.44 \times 100/17.40$, where 17.40 is the amount of CO_2 determined on highly purified calcium pectate (**11**).

The alcohol-insoluble solids in the peel of fumigated and oil-sprayed fruit, respectively, contained, on the average, 5.72% and 5.71% methoxyl (table 1). Since these values are easily determined by saponification with NaOH, a quick method is available for the approximate determination of pectin. The uronic acid–carboxyl groups of pectin are free for salt formation or esterification, but the hemicelluloses do not exhibit acidic properties when treated with alkali at room temperatures. The pectin content of a plant tissue, calculated from the methoxyl values, depends, of course, on the methoxyl content of pure pectin. Since the methoxyl content of pectin varies with the source, method of preparation, etc., an experimental factor has to be determined from a pure pectin prepared by a definite procedure. The methoxyl content of a completely esterified polygalacturonide is 16.32% (**12**), but the use of this figure for calculating a conversion factor would be impractical, since pectins containing such a high concentration of methoxyl have not as yet been isolated (**12**).

The ratio of methoxyl to CO_2 liberated is much lower in grapefuit peel than in lemon peel. In comparison with the CO_2 values of the alcohol-insoluble solids, the methoxyl content of these fractions should be higher than those reported (table 1). This is substantiated by the fact that the pectin equivalent of the CO_2 is greater than that equivalent to the methoxyl content, on the assumption, of course, that pectin contains, on the average, 11% methoxyl. The relatively low methoxyl content and the absence of free acidity indicate that some of the carboxyl groups are combined with the cations of the alcohol-insoluble fraction. This can be shown experimentally by converting the methoxyl values to equivalent percentages of CO_2 plus the CO_2 calculated from the free acidity of the ash-free alcohol-

insoluble solids. For example, the CO_2 that is equivalent to 5.72% methoxyl (average value) is 8.12%; the CO_2 that is equivalent to the free acidity (23.4 mg. NaOH/gm.) of the ash-free alcohol-insoluble solids is 2.57%; the sum of 8.12 + 2.57 = 10.69% total CO_2. This value is in good agreement with 10.44% CO_2, which is the amount determined on the alcohol-insoluble solids by hydrolysis with 12% HCl (table 1).

A comment should be made on the determination of the combined carboxyl groups in the alcohol-insoluble solids. In this fraction the non-esterified carboxyl groups occur in the pectin and are combined with the mineral constituents. The mineral bases were freed from the alcohol-insoluble solids by extracting the latter with $N/10$ HCl in 80% alcohol (**12**). The acidulated alcohol lowers the acidity to pH 1, liberating the carboxyl groups and forming the soluble chlorides of the minerals. After the samples have been washed free of chlorides with 80% alcohol, the free carboxyl groups can be titrated with standard NaOH solution.

The alkaline ash of the alcohol-insoluble solids amounted to an average of 0.780 milli-equivalents per gram of sample. The alkaline ash is a measure of the cations combined with organic substances in the alcohol-insoluble fraction. The substances in combination with the mineral constituents are chiefly pectin and a smaller amount of organic acids that are insoluble in 80% alcohol. Since the alcohol-insoluble fraction contains more than 50% pectin, it is reasonable to expect the mineral constituents to be combined with the non-esterified carboxyl groups, which are equal to 0.584 m.e. per gram. The difference between the alkaline ash and the non-esterified groups is equal to the minerals combined with organic substances other than pectin. In equation form, 0.780 − 0.584 = 0.196 m.e. per gram of alcohol-insoluble solids.

There is an interesting correlation between the calcium content of the alcohol-insoluble solids and the CO_2 that is calculated from an equivalent amount of galacturonic acid anhydride. The alcohol-insoluble fraction contains 0.51 m.e. calcium per gram, corrected for the small amount of oxalic acid combined with the calcium. If the assumption is made that all the calcium is combined with pectin, the following calculation is in order:

176 × 0.51 = 89.76 mg. galacturonic equivalent to 0.51 m.e. calcium per gram alcohol-insoluble solids

89.76 × 0.25 = 22.44 mg. CO_2 equivalent to 0.51 m.e. calcium per gram alcohol-insoluble solids

In other words, 2.24% CO_2 is equivalent to the calcium content of the sample. The sum of the CO_2 that is equivalent to the methoxyl content, plus the CO_2 that is equivalent to the calcium, is equal to the total CO_2 of the fraction. Arithmetically, 8.12 + 2.24 = 10.36% total CO_2. This is in good agreement with the 10.44% obtained on the alcohol-insoluble solids by hydrolysis with 12% HCl.

Pectin content

The composition of the pectin in grapefruit peel, as well as the quantity, is markedly affected by the methods of extraction. For example, the yield (**15, 16**) of pectin from citrus albedo depends upon the pH at which the pectin is extracted and also upon the duration and temperature of the extraction. A standard procedure for the extraction of pectin must be adhered to if reliable results are to be expected. Abundant evidence is available to show that pectins of fruits are not present in the cells and cell walls as fixed and permanent entities but exist

in labile forms that may be transformed with the growth and maturity of the fruit. The composition of pectic acid varies with the treatment received during its preparation.

Since pectic acid is composed of polygalacturonic acids combined with various amounts of *l*-arabinose and *d*-galactose, repeated purification of the material results in a gradual decrease in *l*-arabinose and *d*-galactose, with an ultimate approach to pure galacturonic acid. This makes the quantitative separation and determination of pectin, hemicellulose, cellulose, and lignin a rather complicated and difficult procedure. The polyuronides decompose slowly on hydrolysis with HCl or H_2SO_4 below a concentration of 5%, and the free uronic acids (14) that are formed in the heating are readily decarboxylated.

The pectins were extracted from the alcohol-insoluble solids of the whole peel with hot water and by hydrolysis with dilute HCl. The values were determined as calcium pectate and are reported as percentages of the alcohol-insoluble solids (table 2). The calcium pectates from the water-soluble pectin of the fumigated and oil-sprayed fruit averaged 32.50% and 31.96%, respectively. The calcium pectates from the acid-soluble pectin of the same samples averaged 11.29% and 11.99%, respectively. The total calcium pectate extracted (water-soluble and acid-soluble) was 43.79% for the fumi-

TABLE 2

WATER-SOLUBLE AND ACID-SOLUBLE PECTIN OF ALCOHOL-INSOLUBLE SOLIDS OF GRAPEFRUIT PEEL, DETERMINED AS CALCIUM PECTATE

Sample no.	Total CO_2 (% A.I.S.)	Water-soluble pectin as calcium pectate				Acid-soluble pectin as calcium pectate				Total calcium pectate (% A.I.S.)	Insoluble residue (% A.I.S.)
		Total (% A.I.S.)	Constituents (per cent)			Total (% A.I.S.)	Constituents (per cent)				
			CO_2	Calcium	Furfural		CO_2	Calcium	Furfural		
	Fumigated fruit										
1	10.48	34.33	18.04	8.65	18.45	11.38	17.85	7.82	17.71	45.71	41.10
2	10.35	33.61	18.08	9.18	19.30	11.07	18.20	7.70	17.08	44.68	41.15
3	10.48	30.93	18.45	9.00	18.05	11.01	18.42	8.08	18.13	41.94	41.00
4	10.47	30.98	18.55	9.00	18.70	11.51	18.15	8.00	17.13	42.49	41.20
5	10.43	32.65	18.65	9.02	19.65	11.46	18.37	7.22	17.48	44.11	37.40
Mean	10.44	32.50	18.35	8.97	18.83	11.29	18.20	7.76	17.51	43.79	40.37
	Oil-sprayed fruit										
1	10.48	32.60	18.72	8.91	19.01	11.90	18.07	7.33	17.42	44.50	39.80
2	10.32	31.70	18.40	8.89	19.37	12.06	18.08	7.54	18.07	43.76	40.22
3	10.42	31.60	18.39	9.02	19.00	11.92	17.63	7.77	17.70	43.52	41.21
4	10.37	31.78	18.50	9.02	19.66	11.99	18.47	7.54	18.38	43.77	41.90
5	10.32	32.15	18.41	8.98	19.62	12.07	18.15	7.37	17.33	44.22	41.26
Mean	10.38	31.96	18.48	8.96	19.33	11.99	18.08	7.51	17.78	43.95	40.88

gated fruit and 43.95% for the oil-sprayed fruit.

The degree of purity of the calcium pectate values can be ascertained from their respective CO_2, calcium, and furfural values. The yields of CO_2 from the calcium pectates are slightly higher than the 17.40% (mean) obtained by JOSEPH and BRYANT (11) for highly purified calcium pectates from lemon pectin. The calcium contents are higher than those usually reported for calcium pectate from citrus (6) and from apple pectins (4), and they are higher than would be expected from the CO_2 content—that is, on the basis that calcium pectate should contain 7.45% calcium. GADDUM (6) showed that the calcium content of calcium pectates prepared from pectins which had been repeatedly precipitated with alcohol approached a constant value of 7.50%. In his water-soluble albedo pectins, the initial high calcium values were associated with the presence of high ash. In the present studies the purity of the calcium pectates can be shown by the fact that the alkalinity of the ash is in good agreement with the percentages of calcium.

The uniformity of the calcium content of calcium pectate is evidence that the pectin precipitated by the calcium ion is a chemical substance of considerable uniformity. The claim is frequently and justly made that the calcium pectate method determines the pectin and very few of its decomposition products. The close association of the pectin with the other cell-wall constituents is such that the extraction of the pectin by chemical methods would result in a calcium pectate of variable composition, especially if the extraction processes were prolonged. The calcium pectate method can be used to estimate the pectin content of citrus peel if proper care is used in extraction of the pectin from the tissue. In the present study the calcium in calcium pectate from the water-soluble pectin was higher than that from the acid-extracted pectin (table 2).

The furfural contents of the various calcium pectates are in accord with values reported by other investigators (17) on pectins from other sources, and especially do they agree with the results of BRYANT *et al.* (3) on lemon pectin (*National Formulary*, ed. VIII), which contains a high content of galacturonic acid (87.6%). No claim is made that pectic acid can be freed of arabinose and galactose by precipitation as the insoluble calcium pectate. The complete removal of galactose and arabinose from pectin by hydrolysis (as by HCl in methyl alcohol) results in a degraded form of pectic acid. On the basis of these analyses, the calcium pectate values (table 2) represent, to a considerable degree of accuracy, the pectin content of these extracts.

CELLULOSE AND HEMICELLULOSE FRACTION

The residue that remains after extracting the pectins from the alcohol-insoluble solids (table 2) is composed chiefly of cellulose and hemicellulose (21). This residue contains a comparatively small amount of firmly bound pectin that is difficult to extract and determine quantitatively. As noted in table 2, the percentages of this fraction determined on the different peel samples are somewhat variable, depending, of course, upon the methods used to extract the pectin. The extractants used to dissolve the pectin also dissolve various portions of the hemicellulose. All the CO_2 can be removed from the insoluble residue by prolonged acid hydrolysis, but the calcium pectate determinations made on these hydrolysates are highly contaminated with hemi-

cellulose impurities. The purification of the calcium pectate by redissolving and reprecipitation does not remove all the impurities.

The problem in this study was to adapt methods so as to obtain quantitatively the highest yield of calcium pectate with the least impurities. All the residues that were tested contained CO_2 and thus showed the presence of uronic acids (table 3). After thorough extraction of the alcohol-insoluble solids with hot water and dilute HCl, the residues still contained pectin or uronic acids combined with celluloses or hemicelluloses. The carbohydrate reserve was definitely low, as shown by the absence of starch and dextrin. No substantial amount of evidence exists to prove that the hemicellulose fraction acts as a carbohydrate reserve.

By extracting macerated whole lemons with various solvents, HARVEY (9) found that the ratio of insoluble solids to soluble solids decreased with the advance of maturity. In experiments on stored lemons, he found a steady decrease in the ratio to a minimum near the close of the storage period, followed, generally, by a slight increase. Since most of the insoluble solids are cell-wall constituents, the ratios given for the lemons at different stages of maturity may have been reduced by the increase in soluble solids

TABLE 3

BALANCE SHEET SHOWING RELATION OF CARBON DIOXIDE IN ALCOHOL-INSOLUBLE SOLIDS TO AMOUNT DETERMINED IN CALCIUM PECTATE FROM AQUEOUS AND ACID EXTRACTS

Sample no.	Total CO_2 (% A.I.S.)	CO_2 in aqueous extract			CO_2 in acid extract			CO_2 in residue (% A.I.S.)	Sum of CO_2 fractions determined (% A.I.S.)	Percentage of total CO_2 determined
		Total (% A.I.S.)	Equivalent to Ca pectate in extract (% A.I.S.)	Percentage accounted for	Total (% A.I.S.)	Equivalent to Ca pectate in extract (% A.I.S.)	Percentage accounted for			
	Fumigated fruit									
1	10.48	6.37	6.19	97.17	2.15	2.03	94.42	1.26	9.78	93.32
2	10.35	6.37	6.08	95.45	2.29	2.01	87.77	1.26	9.92	95.85
3	10.48	6.33	5.71	90.21	2.23	2.03	91.03	1.37	9.93	94.75
4	10.47	6.32	5.75	90.98	2.26	2.09	92.48	1.34	9.92	94.75
5	10.43	6.52	6.09	93.40	2.26	2.10	92.92	0.88	9.66	92.62
Mean	10.44	6.38	5.96	93.44	2.24	2.05	91.72	1.22	9.84	94.26
	Oil-sprayed fruit									
1	10.48	6.67	6.10	91.45	2.30	2.15	93.48	1.19	10.16	96.95
2	10.32	6.27	5.83	92.98	2.36	2.18	92.37	1.23	9.86	95.54
3	10.42	6.22	5.81	93.41	2.30	2.10	91.30	1.29	9.81	94.15
4	10.37	6.27	5.88	93.78	2.30	2.21	96.09	1.19	9.76	94.12
Mean	10.40	6.36	5.91	92.91	2.32	2.16	93.31	1.22	9.90	95.19

rather than by an absolute decrease in insoluble solids. This would not be the case in the storage experiments.

With respect to decrease in cell-wall constituents of the peel, the pectin and hemicellulose are metabolized on breakdown of the fruit. Published investigations have established the fact that the rate of pectin decomposition is associated with the keeping qualities of fruits. In the lemon fruit the soluble pectin increases as the fruit changes from green to mature, either on the tree or in storage. The rate of change from insoluble to soluble pectin is a function of the temperature. Total pectin, both soluble and insoluble fractions, decreases if the storage period is prolonged or if the fruit remains on the tree an excessive period after maturity.

Distribution of carbon dioxide in the different fractions of the alcohol-insoluble solids

The degree of efficiency with which the calcium ion precipitates pectin in grapefruit peel can be observed from the data in table 3 by comparing the CO_2 contents of the various extracts with the corresponding CO_2 values of the calcium pectates. For the fumigated and oil-sprayed samples, the mean amounts of CO_2 in the aqueous extracts were, respectively, 6.38% and 6.36% of the alcohol-insoluble solids. The corresponding CO_2 values for the calcium pectates prepared from these extracts were 5.96% and 5.91%. In the two groups of samples, the amounts of CO_2 in the calcium pectates accounted for 93.44% and 92.91% (mean values) of the total CO_2 in the aqueous extracts.

Experiments similar to those on the aqueous extracts were performed on the acid extracts of the alcohol-insoluble solids. In these experiments the amounts of CO_2 in the calcium pectates accounted for an average of 91.72% and 93.31% of the total CO_2 in the acid extracts.

The preceding results show that more than 90% of the uronic acid-CO_2 extracted from the alcohol-insoluble solids with water and dilute acid solution was precipitated as calcium pectate. Undoubtedly, a portion of the loss in CO_2 occurred in the large volume of wash water used to free the calcium pectate of chlorides. The fact that most of the CO_2 occurred in the galacturonic acid of the calcium pectate indicates that only a small amount of uronide, if any, could be combined with the hemicellulose dissolved by the water and dilute HCl solutions in the extraction process.

Another point worthy of note is the relation of the CO_2 of the alcohol-insoluble solids to the sum of the CO_2 of the various fractions. As shown in table 3, the sum of the CO_2 in the aqueous extract, acid extract, and residue averaged 9.84% and 9.90% of the alcohol-insoluble solids (means of the two sample groups). The latter values are equivalent to 94.26% and 95.19% of the total CO_2 content of the alcohol-insoluble solids. Thus, approximately 5% of the total CO_2 was lost in the extraction processes.

Comments

Harvey and Rygg (10) did not find pectic acid in either the flavedo or the albedo of grapefruit peel. The high pH value of the peel and the presence of relatively high concentrations of cations would indicate that any free carboxyl groups of the pectin would react with the metallic bases to form salts. The fact that the sum of the CO_2 equivalent to the methoxyl content and the CO_2 equivalent to the non-esterified groups is equal to the total uronic acid-CO_2 is evidence that free carboxyl groups of pectin are not present in macerated grapefruit peel.

RYGG and HARVEY (19) noted that fluctuations in the total pectin content of the albedo were due to the effect of environmental changes on the fruit rather than to the effect of seasonal trends. The total pectic substances in their samples ranged from 14.5% to 18.9% calcium pectate (dry-weight basis), with the minimum quantity occurring in midseason and the maximum at the end of the season. As noted by RYGG and HARVEY (19) and by SINCLAIR and BARTHOLOMEW (20), this variability is not surprising, in view of the fact that the climatic environments in which grapefruit is grown markedly affect its composition.

The accuracy of some of the methods used in the present investigation is not of the highest order, but the procedures finally adopted proved to be the most satisfactory for the experimental conditions. The fact that the solubility of pectin depends upon the pH, the temperature, and the duration of the extraction is sufficient evidence that a given procedure must be defined and strictly followed if comparable results are to be obtained. An analyst thoroughly familiar with the methods and material can determine the pectins in the alcohol-insoluble solids of grapefruit peel with an error of less than 5%. The more empirical a method is, the more necessary it is to follow a standard procedure.

The methods used in this study for the extraction and determination of pectin are concerned chiefly with the total amount of pectin rather than with a product having the physical and chemical properties so essential for a commercial grade of pectin. No claim is made that these methods are suitable for the commercial extraction of pectin, but they are useful for the quantitative estimation of pectin on small laboratory samples. From a biochemical standpoint, the relation of the total pectin to the other carbohydrate constituents of the peel is of importance because some of the pectin in the peel is closely associated with the cellulose and hemicellulose fractions. The methods that are used, therefore, not only must be quantitative but must yield a product with the least possible degradation.

Summary

1. Whole peel of grapefruit was extracted with 80% ethyl alcohol and separated into two distinct fractions, the alcohol-soluble and alcohol-insoluble solids. The carbohydrate constituents of these two fractions were investigated, and certain of their physical and chemical properties were determined. All analyses were made on fruit samples obtained from trees that had been fumigated with hydrocyanic acid or sprayed with oil for pest control during the growing season.

2. The alcohol-soluble fraction of the peel contained substances which liberated a small amount of carbon dioxide on hydrolysis with 12% hydrochloric acid. The total solids of this fraction composed 66.31% and 67.35% of the dry matter of the peel of fumigated and oil-sprayed fruit, respectively.

3. The soluble sugars in the peel averaged 40.29% and 41.28% of the dry weight of the fumigated and oil-sprayed fruit, respectively. These amounts of soluble sugars accounted for 60.76% and 61.28% (mean values) of the total alcoholic extractives. Approximately 40% of the dry weight of the alcohol-soluble fraction was composed of substances other than sugars, namely, essential oil, waxes, organic acids, naringin, and various concentrations of undetermined constituents.

4. The alcohol-insoluble fraction of the peel contains the cell-wall constituents—

cellulose, hemicellulose, and pectin. This fraction comprised 33.69% and 32.65% of the dry weight of the peel. Lignin and starch were not present in sufficient quantities to be determined by the prevailing chemical methods.

5. Since the alcohol-insoluble fraction contains the pectin, most of the carbon dioxide which can be liberated by hydrolysis with 12% HCl and all the methoxyl groups occur in this fraction. The ratio of methoxyl to carbon dioxide was much lower in grapefruit peel than in lemon peel.

6. The carbon dioxide equivalent to the sum of the esterified and non-esterified carboxyl groups was equal to the total carbon dioxide of the alcohol-insoluble fraction obtained on hydrolysis with 12% hydrochloric acid.

7. The sum of the water-soluble and acid-soluble pectin (as calcium pectate) of the alcohol-insoluble fraction of the peel of the fumigated and oil-sprayed fruit amounted to 43.79% and 43.95%, respectively. These values are much lower than those for the total pectin calculated from the carbon dioxide and the methoxyl values.

8. To establish criteria of purity, the calcium pectate values are accompanied by corresponding values for carbon dioxide, calcium, and furfural. The percentages of calcium in the calcium pectates are somewhat higher than the 7.50% usually reported for pure calcium pectate. The percentages of carbon dioxide are slightly higher than the 17.40% reported for pure calcium pectate. The furfural values are in accord with those reported by other investigators.

9. The residue that remains after extracting the pectin from the alcohol-insoluble solids of the peel is composed of cellulose and hemicellulose and a comparatively small amount of firmly bound pectin that is difficult to extract and determine quantitatively.

10. The sum of the carbon dioxide in the aqueous extract, acid extract, and residue amounted to 94.26% and 95.19% of the total carbon dioxide of the alcohol-insoluble solids in the peel of fumigated and oil-sprayed fruit, respectively. In the aqueous and acid extracts, calcium pectate accounted for more than 90% of the carbon dioxide dissolved by the extractants.

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FACTORS AFFECTING THE DESTRUCTION OF CAROTENE IN ALFALFA

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 610

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Introduction

The importance of enzymes, light, temperature, and oxygen as factors in the destruction of carotene in alfalfa and other plant materials has been studied by many workers. From their studies on stability of carotene in grass during haymaking and storage, SESHAN and SEN (13) concluded that oxygen is the principal factor and that light, enzymes, and temperature merely serve as accelerators. They did not explain, however, a 50% loss of carotene in a sample dried *in vacuo* at 31° C. when no loss was observed in blanched samples dried under similar conditions. BERNSTEIN and THOMPSON (2), in their studies of carotene-destroying processes in bean leaves, concluded that loss of carotene during dehydration is a combination of enzymatic action and photodestruction, with both processes being influenced by oxygen pressure. Of these two factors, enzymatic destruction did not occur below an oxygen pressure of 0.02% oxygen, while decreasing the oxygen below 0.5% did not further decrease the photodestruction. They suggested that some cellular constituents may replace oxygen in the photodestruction of the carotenoid pigments. They also found that the wave length of the light had no significant effect on the destruction of carotene but that the loss was roughly proportional up to 300 foot-candles. MITCHELL and co-workers (6, 7) considered enzymatic destruction of carotene in alfalfa to be of primary importance, while GUILBERT (3) emphasized

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the importance of light. The destruction of carotene by the lipoxidase-fat system described by SUMNER (**14, 15**) has been widely studied, and MITCHELL and HAUGE (**6**) presented evidence to show that such a lipoxidase-fat system may be responsible for the enzymatic destruction in alfalfa.

The role of chlorophyll as a light-sensitizing agent in various oxidations and reductions *in vitro* and *in vivo* was reviewed by RABINOWITCH (**12**). ARONOFF and MACKINNEY (**11**) demonstrated that chlorophyll can destroy carotene in solution. KIRSANOVA (**4**) found that ultraviolet light destroys carotene rapidly in aqueous colloidal solutions and in petroleum ether extracts but in most cases does not affect it in vegetable juices. PEPKOWITZ (**10**) reported that acetone and petroleum ether extracts of vegetables which contained chlorophyll lost carotene when exposed to light, the amount of loss being related to the length of exposure, but that xanthophylls and flavones did not produce such results. Further work by this investigator (**11**) showed that oxygen was required for the photodestruction of carotene, but in very small amounts, and that an increased oxygen concentration had little effect. MITCHELL and KING (**8**) found that sunlight could cause appreciable loss of carotene when alfalfa extracts were exposed but that exposure to ultraviolet or Mazda lamps placed 1–3 feet, respectively, from such extracts did not result in appreciable destruction of carotene.

The studies herein reported were made to obtain further data on the effect of enzymes, light, and oxygen on the loss of carotene in drying alfalfa and to investigate the factors which caused blanched alfalfa stems to bleach rapidly when exposed to light for a short time. The possibility of using the destruction of carotene in extracts and purified solutions as an indicator to aid in the isolation of natural antioxidants is considered.

Material and methods

The alfalfa used in this work was grown near Riverside, California. In all cases plants selected were at least 10 inches in height and had not bloomed. In most cases they were cut and brought to the laboratory immediately, and the top 8 inches were used for the tests. Since almost complete bleaching of blanched, sun-dried stems had been observed previously, stems and leaves were considered separately. Stem samples consisted solely of the central stems, while "leaf" samples usually consisted of leaflets, petioles, and stipules, the separation being made by hand-picking. Since the petioles seemed to be intermediate between stems and leaves in response to the factors studied, it should be recognized that they probably exert a modifying influence and that the response of the so-called "leaf" fraction to these factors may not be the same as would be given by leaflets alone.

Unless otherwise specified, blanched samples were blanched with steam in a pressure cooker at 5 lb./sq. in. (108° C.) for 1 minute.

In studies of carotene loss during dehydration the samples were exposed to the experimental conditions until dry. In some cases there were marked differences in the lengths of exposure when using this method. Although such differences may have resulted in more or less loss, losses during dehydration and after dehydration are considered as separate processes even though they may be affected by the same factors.

Exposure to light was made under a variety of conditions. When sunlight is specified, samples were usually exposed

from 12:00 to 15:00 hours P.D.T. during the period October 15–November 15, 1948, on bright, clear days. Vacuum-drying was accomplished with an oil vacuum pump which gave a vacuum of 0.01–0.1 mm. of mercury. In most cases such drying was carried out in an oven at 60° C., but, when vacuum drying in the sun, samples were placed in a desiccator which was evacuated constantly with a vacuum pump.

Green samples usually weighed 10–15 gm. and were run in triplicate or quadruplicate. After drying, the samples were ground to 40 mesh in a small Wiley mill. Carotene determinations were made using the method of ZSCHEILE and WHITMORE (**17**) on a dried sample of 1–2 gm. In the purified systems carotene was separated chromatographically prior to spectrophotometric readings. A Klett photoelectric colorimeter with a blue filter calibrated with pure β-carotene was used in all determinations of carotene. All data are presented as the β-carotene equivalent, without regard to the isomer-content influence on the results. Carotene losses were calculated on the basis of 100% retention in blanched samples dried in a vacuum oven in the dark or, in the case of the purified systems, on the initial carotene content.

Experimentation and results

CAROTENE DESTRUCTION DURING DRYING

To determine the loss of carotene in blanched and unblanched stems and leaves in the presence and absence of sunlight, samples were exposed to sunlight or dried in a forced-draft oven at 65° C. Leaf samples placed in sunlight or oven were dry in 2 hours, as were blanched stem samples in the oven. The blanched and unblanched stems were not dry after exposure to sunlight from 13:00 to 16:00 P.D.T., and the drying was finished under clear infrared lamps overnight. Carotene loss is shown in table 1. The carotene content of blanched vacuum-dried samples is assumed to be 100%.

From a comparison of the losses in the leaf samples, it seems probable that sunlight caused 7–8% of the carotene loss in drying and enzymes a loss of 27–28%. With the stems, however, it was impossible to determine how much loss was caused by enzymes and how much by light. In the unblanched samples the decrease of the original carotene was only 5% more in the light- than in the oven-dried samples; it would seem that enzymatic destruction under these conditions probably amounted to as much as 70%. Because the losses in the blanched samples dried in the oven or sun were so large, however, no valid conclusions can be made of the extent of enzymatic or photodestruction. It is evident from these data that it would be impossible to determine the extent of enzymatic or photodestruction in whole plants, using the techniques of this experiment, because the carotene in stems seems to be so unstable and because there is such a marked difference between stems and leaves.

The difference between stems and leaves was further emphasized in an experiment in which leaf and stem fractions were dried under different kinds of light bulbs. In the stem fractions the stipules were left attached. For the experiment, 10-gm. samples of leaves and 15-gm. fractions of stems were placed 20 inches below 250-watt G.E. infrared bulbs, both clear and amber-colored, and a 400-watt mercury-vapor lamp. Light intensities were determined with a Weston meter and are approximations only.

The experimental details and results are given in table 2. Carotene loss in the stems dried under lamps was quite large. If it is assumed that 14% of the loss of carotene would have occurred during drying in the dark (oven-dried sample of table 1), and that the light intensities given in the table are approximately correct, it is evident that very little light is required to cause an appreciable increase in the loss of carotene. The loss, however, seems to be roughly proportional to the light intensity from 18 to 1200 foot-candles. Measurements of light intensity under the mercury-vapor lamp were made by interposing a screen between light meter and lamp and multiplying the result by the screen factor. The ultraviolet filter described below reduced the light intensity from 2400 to 1600 foot-candles. Since this lamp, which was rich in ultraviolet, and the clear infrared lamp (1200 ft-c.), which was low in ultraviolet rays, caused about the same loss of carotene, it is probable that ultraviolet light is relatively ineffective in destroying carotene during sun-drying.

This fact is also indicated by an experiment in which blanched alfalfa stems and leaves were dried in the sun under an ultraviolet filter or exposed to full sunlight. The filter was prepared by spreading a layer of cellulose nitrate containing Michler's ketone (dissolved in acetone and ethyl cellosolve) over 8-inch squares of single-weight windowpane. Transmission measurements with a Beckman spectrophotometer indicated less than 1% light transmission in the band 310–400 mμ and about 80% transmission at 460–800 mμ. Carotene loss during drying under the filter was 25 and 90%, respectively, for leaves and stems, while losses in the sun-dried samples were 28 and 92%. Since the filter absorbed at least 20% of the light throughout the visible spectrum, it is conceivable that the small difference observed between the readings could have been the result of less visible light transmitted through the filter rather than of the filtering-out of the ultraviolet.

Since a 14% loss of carotene occurred in blanched stems which were dried in the dark, it was obvious that light as such was not the dominant factor in caro-

TABLE 1

CAROTENE CONTENT (P.P.M.) OF BLANCHED AND UNBLANCHED ALFALFA STEMS AND LEAVES DRIED IN SUNLIGHT AND IN FORCED-DRAFT OVEN AT 65° C.

EXPERIMENTAL CONDITIONS	LEAVES						STEMS					
	1	2	3	4	Av.	% loss	1	2	3	4	Av.	% loss
Blanched, vacuum-dried at 60° C.	506	490	490	495	495	0	83	77	90	83	83	0
Unblanched:												
Sun-dried	298	302	297	309	302	39	21	22	20	.....	21	75
Oven-dried	*309*	*341*	*362*	*357*	*342*	*31*	*27*	*18*	*31*	*22*	*25*	*70*
Blanched:												
Sun-dried	442	453	436	436	442	11	2	4	3	.....	3	96
Oven-dried	479	479	495	452	476	4	69	64	78	72	71	14

tene destruction. To study the possibility that this loss was purely oxidative in nature, blanched and unblanched stems were dried in sunlight and in the dark *in vacuo*. In this experiment the blanched stems were placed in a vacuum desiccator in the sun at 25° C. for 2 days to insure complete drying before opening, while the samples in the dark were dried in a vacuum oven at 60° C. overnight. The unblanched samples which were exposed to the sun were placed in the vacuum desiccator, spread out on black paper, while the unblanched samples which were dried in the dark were placed in telescoped, opaque, black envelopes in the same desiccator. Blanched samples were dried in a vacuum oven overnight to serve as a control. The unblanched samples were dried under continuous vacuum for 2 days under conditions very similar to those of the blanched samples. The results for both experiments are given in table 3. The insignificant loss of 2 and 3% of the carotene in the unblanched and blanched stems, respectively, during vacuum-drying in sunlight gives conclusive proof that all carotene loss, whether light-sensitized or enzymatic, is dependent on the presence of oxygen.

To determine the possible effect of water-soluble substances on carotene stability during drying, leaf and stem samples were blanched, pressed to 10,000 lb./sq. in. in a Carver press, soaked in water, and re-pressed to 10,000 lb./sq. in. The press cake was separated and dried as indicated in table 4. The stem samples listed as sunlight samples were dried in the sunlight for 4 hours and overnight under the clear infrared lamps. A comparison of these results with those given in table 1 indicated that water-soluble substances in the leaves decreased the light-sensitized loss of carotene but played little part in the carotene loss in the dark. Although the loss of carotene in pressed stems was much less than in unpressed stems, whether dried in sunlight or in darkness, a part of the difference was probably the result of more rapid drying in the pressed stems.

TABLE 2

CAROTENE CONTENT (P.P.M.) OF BLANCHED STEMS AND LEAVES DRIED UNDER INFRARED AND MERCURY-VAPOR LAMPS

Light treatment	Leaves						Stems					
	1	2	3	4	Av.	% loss	1	2	3	4	Av.	% loss
Vacuum-dried control	442	436	422	426	432	0	120	114	126	125	121	0
Clear, infrared (approx. 1200 ft-c.)	414	420	386	414	408	6	55	52	55	.....	54	55
Amber, infrared (approx. 400 ft-c.)	422	430	406	.....	420	3	75	78	69	.....	74	39
Amber, infrared with deep red filter (approx. 18 ft-c.)	418	416	398	.....	408	6	76	95	.....	.....	86	29
Mercury vapor (2400 f-c., 800 ft-c. removable in u.v. filter)	386	402	402	.....	396	8	49	55	.....	.....	52	57

Many different blanching methods have been reported in the literature. SESHAN and SEN (13) used 2 atmospheres for 1 hour, and BERNSTEIN and THOMPSON (2) used 5 lb./sq. in. for 5 minutes; the practice in this laboratory was to blanch for 1 minute at 5 lb./sq. in. To determine the effect of blanching conditions on the carotene content of samples dried at 65° C. in the dark, 25-gm. samples of whole alfalfa were blanched at 5 lb./sq. in. for 1, 5, 30, and 60 minutes. It is evident from the data (table 5) that the blanching conditions tried had no

TABLE 3

CAROTENE CONTENT (P.P.M.) OF BLANCHED AND UNBLANCHED ALFALFA STEMS DRIED IN VACUO IN DARK AND IN SUNLIGHT

Drying conditions	1	2	3	4	Av.	% loss
Blanched, oven-dried	99	109	109	121	110	0
Blanched, sunlight	109	104	103	112	107	3
Blanched, oven-dried	101	101	103	101	102	0
Unblanched, dark	88	96	92	95	93	9
Unblanched, sunlight	96	104	100	98	100	2

TABLE 4

CAROTENE CONTENT (P.P.M.) OF BLANCHED AND PRESSED ALFALFA LEAVES AND STEMS DRIED IN DARK AT 65° C. AND IN SUNLIGHT

Drying conditions	Leaves						Stems					
	1	2	3	4	Av.	% loss	1	2	3	4	Av.	% loss
Vacuum-dried in dark	620	599	575	569	591	0	122	117	120	111	118	0
In sunlight	440	495	508	472	479	19	39	45	47	43	44	63
In dark	575	579	600	543	574	3	109	110	111	120	112	5

TABLE 5

CAROTENE CONTENT (P.P.M.) OF ALFALFA BLANCHED AT 5 LB./SQ. IN. FOR VARYING LENGTHS OF TIME AND DRIED IN OVEN AT 65° C.

Replicate	Minutes blanched				
	1*	1	5	30	60
1	429	364	371	384	412
2	401	396	362	358	326
3	397	368	380	363	374
4	382	404	373	418	424
5	350	375	412	394	423
6	361	378	395	372	384
7	387	387	387	407	416
8	407	400	355	403	415
Av.	389±9	384±9	379±7	387±8	398±12

* Vacuum-dried.

significant effect on the carotene content of the dried material. There was very little difference in the appearance of the samples blanched for 1 and 5 minutes, but the others were brown and very soft, indicating destruction of chlorophyll and loss of leaf and stem structure. The carotene stability during storage, however, is dependent on the blanching conditions. In an accelerated storage test with the samples stored at 65° C. for 10 days, the loss of carotene in the samples blanched for 1, 5, 30, and 60 minutes was 68, 76, 79, and 78%, respectively. These data are presented in table 6 with storage data from some of the experiments given above. In all cases the percentage loss is calculated from the carotene content after drying rather than from the original carotene content of the tissue.

From the data the following conclusions seem valid. (*a*) Part of the antioxidants naturally present are heat labile and are destroyed by prolonged blanching. (*b*) Blanching followed by pressing removes natural antioxidants active in stabilizing carotene during storage in leaves, but the same conditions have little effect on the stability of carotene in stems. (*c*) Carotene in leaves is more stable than in stems during storage as well as during drying—a further indication of more natural antioxidants in leaves.

TABLE 6

DESTRUCTION OF CAROTENE DURING STORAGE AT 65° C. FOR 10 DAYS

Treatment	% loss
Whole alfalfa blanched at 5 lb./sq. in. for:	
1 minute	68
5 minutes	76
30 minutes	79
60 minutes	78
Blanched leaves	65
Blanched stems	87
Blanched, pressed leaves:	
Vacuum-dried	77
Dried in oven at 65° C.	74
Dried in sunlight	76
Blanched, pressed stems:	
Vacuum-dried	88
Dried in oven at 65° C.	93
Dried in sunlight	88

PURIFIED SYSTEMS

To study the nature of the antioxidants involved in carotene stabilization, it was necessary to use purified systems and to carry out isolation procedures.

An acetone extract was made of leaves and stems, and the pigments were transferred to Skelly Solvent B. Water-soluble substances and acetone were removed by continuous washing. Dilutions were made of the leaf and stem extracts so that each dilution contained 39.6 μgm. of carotene in 5 ml. Five-ml. portions of the diluted solutions were pipetted into 2-oz. prescription bottles, and the bottles were tightly capped with foil-lined screw caps. These were then placed flat-side down on a rack about 1 cm. above a 36-inch fluorescent tube. The light intensity with this arrangement amounted to about 600 foot-candles. With three such tubes a total of twenty-four samples could be irradiated at one time. A purified system containing 558 μgm. of chlorophyll *a*, 186 μgm. of chlorophyll *b*, and 42.4 μgm. of carotene per 5 ml. was run at the same time for comparative purposes. Crystalline β-carotene was used while chlorophylls *a* and *b* were prepared from alfalfa by the method of ZSCHEILE and COMAR (**16**). Chlorophyll concentrations were calculated from absorption data at the red maxima as determined on a Beckman spectrophotometer. Duplicate samples of each solution were removed from the irradiating apparatus at 20-minute intervals, and the carotene was separated from the chlorophylls chromatographically. It is

obvious from the data (table 7) that both stem and leaf extracts contained some substance or substances that prevented the light-sensitized oxidation of carotene. The average rate of loss calculated as the percentage loss in 20 minutes is one and a half times greater in the stem extract than in the leaf extract.

To show the effect of concentration of pigments on the rate of carotene destruction, another leaf extract was made and were confirmed qualitatively in the following experiment. Solutions containing 41.1 μgm. of carotene per 5 ml. and varying concentrations of purified chlorophyll *a* were exposed to light for 30 minutes. The percentage loss of carotene as a function of the original chlorophyll concentration is presented in figure 1. It is interesting that only very low concentrations of chlorophyll are required for significant carotene destruction.

TABLE 7

CAROTENE STABILITY OF LEAF AND STEM EXTRACTS DURING EXPOSURE TO LIGHT COMPARED WITH SOLUTION OF PURIFIED CHLOROPHYLL AND CAROTENE

CAROTENE CONTENT IN μGM./5 ML.

MINUTES	PURIFIED			STEM EXTRACT			LEAF EXTRACT		
	Carotene	Average	% rem.	Carotene	Average	% rem.	Carotene	Average	% rem.
0	42.4			39.6			39.6		
0	42.4	42.4	100	39.6	39.6	100	39.6	39.6	100
20	31.8			33.2			35.8		
20	30.9	31.4	74	33.2	33.2	84	38.0	36.9	93
40	19.6			28.1			32.6		
40	20.8	20.2	48	27.5	27.8	70	31.8	32.2	81
60	18.3			23.6			29.0		
60	16.6	17.5	41	24.9	24.2	61	28.6	28.8	73
80	8.5			18.3			26.8		
80	12.8	10.7	25	21.1	19.7	50	25.8	26.3	66
Av. % loss in 20 minutes		35.8			15.9			10.4	

diluted to give carotene contents of 21, 53, and 146 μgm. of carotene per 5 ml. When the samples were irradiated, the rate of loss calculated on the same basis was 20, 13.5, and 8% for 20 minutes, respectively, for the three dilutions. Accordingly, the pigment concentration must be considered in evaluating the results from such experiments, because the rate of loss was much less in the more concentrated extracts.

The effect of the chlorophyll-carotene ratio on carotene destruction has been reported by PEPKOWITZ (11) and ARONOFF and MACKINNEY (1). The results

The addition of compounds known to be antioxidants for alfalfa meal to pure solutions of chlorophyll and carotene did not decrease the carotene loss during exposure to light. Compounds tested were catechol, isopropoxy diphenylamine, and soybean tocopherols.

Since chlorophyll had been shown by MEYER (5) to sensitize the photo-oxidation of oleic acid, the effect of varying concentrations of purified ethyl linoleate was tried. For the experiment the carotene and total chlorophyll concentration in 5 ml. were adjusted to 48.1 and 263 μgm. (195 μgm. chlorophyll *a*, 68 μgm.

chlorophyll *b*), respectively, while the ethyl linoleate concentration was varied from 10 to 2560 μgm. At the end of a 30-minute exposure the control solution had lost 29% of the original carotene, while those containing ethyl linoleate had lost 34–37%. The concentration of the ester had no significant effect. Under these conditions ethyl linoleate had little effect on carotene destruction and certainly did not compete with carotene for the oxidizing potentialities of the chlorophyll. Also, it seemed unlikely that an oxidizing system similar to the lipid-lipoxidase system was operating.

A leaf extract was fractionated chromatographically on a column of powdered sugar and was separated into four zones, namely: (*a*) carotene, (*b*) luteol, (*c*) chlorophyll *a*, and (*d*) chlorophyll *b*. All the zones contained substances other than those designated, and the presence of some carotenols in the chlorophyll fractions was visibly evident. The chlorophyll and carotene contents of these fractions were adjusted to equal concentrations by the addition of purified chlorophylls *a* and *b* and carotene where necessary. The stability of the carotene during irradiation for 1 hour was determined. A solution of pure chlorophylls *a* and *b*, carotene, and the original extract was run for comparative purposes. The losses as percentages of the initial carotene were 68 for pure chlorophyll and carotene, 64 for the carotene fraction, 60 for the luteol fraction, 58 for the chlorophyll *b* fraction, 52 for the chlorophyll *a* fraction, and 31 for the original extract. It is evident that the naturally occurring antioxidants were distributed in all the fractions but were most concentrated in the chlorophyll *a* fraction. Unfortunately, time did not permit more detailed separations and tests using the above technique. It is thought, however, that this approach may lead to the isolation of the natural antioxidants for the light-sensitized oxidation of carotene as well as the natural antioxidants for carotene destruction in storage.

Discussion

This work confirms the conclusions of others (**2, 13**) that carotene destruction is truly oxidative in nature, whether enzymatic, light-sensitized, or influenced by other factors. The difference between leaf and stem tissue is one that does not seem to have been recognized previously but one that should be considered in all work concerned with the destruction of carotene in alfalfa. This may be true for other plants as well.

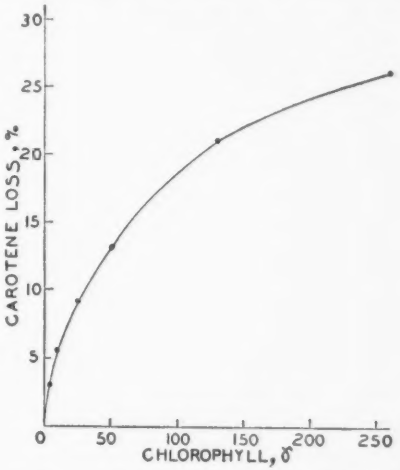

FIG. 1.—Effect of increasing concentrations of chlorophyll *a* on photodestruction of carotene.

Although the results presented above are inconclusive, the following tentative hypotheses are advanced to explain some of the observations. Stems contain less or none of an antioxidant or antioxidants present in leaves. The carotenols may play a part in the greater stability of both stem and leaf extracts in comparison with purified chlorophyll and caro-

tene solutions. NOACK (9) has shown that carotenols as well as carotene are destroyed in such systems and that chlorophyll is protected from bleaching. In this connection, carotenols may retard carotene destruction by competing with carotene for the oxidizing potentialities of the chlorophyll. Because the ratio of chlorophyll to carotene and carotenols is essentially the same in stems and leaves, however, it is doubtful if the carotenols are responsible for the difference found in the stability of carotene in extracts, although it is possible that a difference in esterification might bring about greater stability in the leaf extracts.

The fact that antioxidants which are known to be active in stabilizing carotene in storage had no effect on the light-sensitized loss of carotene in the purified systems indicates that natural antioxidants for these systems may be different.

The evidence that ultraviolet light had little if any effect on carotene destruction is contrary to the usually accepted idea of the destructiveness of ultraviolet light. The evidence is admittedly indirect and should be subjected to a more thorough study. The work of KIRSANOVA (4), however, who found that ultraviolet light did not destroy carotene in extracts, should be considered.

Summary and conclusions

1. Oxygen is necessary for carotene destruction in alfalfa, and other factors act as accelerators.

2. There is a basic difference in the stability of carotene in alfalfa stems and leaves, the carotene being much more stable in leaves.

3. Sunlight-sensitized loss of carotene in detached leaves is 7–8%, while the enzymatic loss is about 27–28%.

4. In unblanched stems sunlight-sensitized loss of carotene is probably of the same order of magnitude as that in leaves, but the enzymatic destruction may be as much as 70%.

5. Ultraviolet light is relatively unimportant in carotene loss in alfalfa during drying.

6. Blanching for long periods destroys natural antioxidants and decreases the carotene stability during storage.

7. Chlorophyll acts as a light-sensitizing agent for carotene oxidation in solutions and is probably responsible for the light-sensitized loss of carotene in sun-drying.

8. Evidence is presented that the natural antioxidants may be isolated by fractionation of the extracts.

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USE OF TOMATO JUICE IN THE ASYMBIOTIC GERMINATION OF ORCHID SEEDS

EMIL F. VACIN AND F. W. WENT

Introduction

The history of the germination of orchid seeds is an interesting one. In the middle of the nineteenth century commercial growers were germinating orchid seeds; their methods, kept as trade secrets, probably simulated the natural jungle environments of the orchids. In the jungles seedling orchids are found only rarely, usually at the bases of mature orchid plants. The difficulties for germination of the seeds in their natural habitat can be best appreciated after an examination of an orchid seed pod which contains thousands of powdery seeds. Although estimates indicate that each pod of a *Cattleya* species contains approximately 500,000–750,000 seeds, they rarely germinate.

Not until the early part of the twentieth century did botanists concern themselves with the problem of germinating orchid seeds, when BURGEFF (**3**) in Germany and BERNARD (**1**, **2**) in France reported that orchid seeds possessed insufficient food for germination. Upon examination of the germinating seed they found a fungus within the embryo, identified by BERNARD as *Rhizoctonia*. Both authors postulated that a symbiotic condition existed. Commercial growers quickly used this information to devise various methods of germination involving the use of *Rhizoctonia*. Even with the improved methods, however, the results obtained were not consistently successful.

In 1922 KNUDSON (**4**) introduced an ingenious method of germination which did not require the use of *Rhizoctonia*. He found that the addition of sugar to the culture media resulted in consistent germination. He continued his work (**5**) and in 1925 published (**6**) the results of his physiological studies of symbiotic germination of orchid seeds, proving beyond a doubt that germination can be accomplished without the use of the fungus and that the fungus probably did not act as a symbiont.

KNUDSON'S sterile-culture method of germinating orchid seeds was not difficult, and most commercial growers soon began to use it with great success. This so stimulated hybridization that in the last quarter of a century the resultant hybrid blooms far surpass in beauty and size those of the natural orchids growing in their jungle environments. Moreover, this also encouraged the culture of orchids by the amateur grower.

Further to facilitate the germination

of orchid seeds, a definite need for further simplification of the KNUDSON sterile-culture medium seemed, to the writers, to be desirable. They postulated that if the orchid embryo has insufficient food for germination, additional food might be supplied by an agar medium supplemented with fruit juices. In 1944 the writers experimented with persimmon- and tomato-juice extracts. In 1945 MEYER (8) published a paper on the use of tomato juice as a nutrient in the germination of orchid seeds, in which he indicated the need for further study of the problem.

Because of the interest in the use of tomato juice as the basis of a nutrient solution, experiments have been conducted by the writers since 1944.

Experimental procedures and results

PREPARATION OF TOMATO-JUICE SOLUTIONS.—These tomato-juice solutions differ somewhat from those used by MEYER (8) in that different concentrations of the juice were used; in addition, since the carbohydrate content in tomatoes varies, sucrose was added to assure a sufficient supply of sugar. The following formula was used:

Tomato juice	750 ml.
Distilled water	250 ml.
Sucrose	10 gm.
Agar	15 gm.

When vine-ripened tomatoes were available, they were picked, immediately quartered, mashed in a colander, and the resultant juice with the pulp was strained through a sieve, having twelve meshes per inch, to remove the seeds and pulp. This strained juice was called solution 2. A filtered sample was prepared by passing solution 2 through no. 1 Whatman filter paper. The resultant clear amber solution was designated solution 1. Large quantities of both solutions were prepared at one time, placed in jars, and autoclaved. This procedure assured uniform juice for extensive experiments.

DETERMINATION OF BUFFER ACTION OF TOMATO-JUICE SOLUTIONS.—When tomato-juice solutions were used, a considerable volume of *N* KOH was required to raise the pH value from 4.14 to 5.50, indicating a strong buffering action. Thus, it seemed desirable to determine the buffering action of these solutions before any experiments involving them were begun. Therefore, freshly prepared strained tomato juice (solution 2) without agar was tested for its buffering action. To obtain comparative data, KNUDSON's solution C (7) was also tested. Solution C differs from his previously reported solution B (4) in that the $FePO_4$ of solution B is replaced by $FeSO_4$, and $MnSO_4$ is added.

SOLUTION C

$Ca(NO_3)_2 \cdot 4\ H_2O$	1.000	gm
KH_2PO_4	0.250	gm.
$MgSO_4 \cdot 7\ H_2O$	0.250	gm.
$(NH_4)_2SO_4$	0.500	gm.
$FeSO_4 \cdot 7\ H_2O$	0.025	gm.
$MnSO_4 \cdot 4\ H_2O$	0.0075	gm.
Sucrose	20.0	gm.
Agar (flake)	18.0	gm.
Distilled water	1000.0	ml.

The buffer curves are shown in figure 1. The curve for solution B is not indicated but was practically identical with that of solution C. The unfiltered tomato juice (solution 2) definitely showed a very strong buffering action, while solution C was weakly buffered. It is generally recognized that a satisfactory pH range for good growth is pH 4.5–5.5 (9), as indicated in figure 1. Within this range solution 2 was buffered about fifty times as strongly as solution C. VACIN and WENT (9) have shown that, when solution C is buffered more strongly with 0.6% KH_2PO_4 in place of 0.025%, the buffer-

ing action is increased only four times (fig. 1). Experiments now in progress indicate, however, that this more strongly buffered solution C is toxic to, or at least less effective for, orchid seeds. This will be discussed in another paper.

EFFECT OF AUTOCLAVING ON PH VALUE OF TOMATO-JUICE SOLUTIONS.—It has been shown (9) that solution C without agar, adjusted to various pH values, becomes more acid upon autoclaving, having two maximal deviations at pH 5.5 and 8.0. It was found that, with the initial values between pH 3 and 6, the increase in acidity resulted from the presence of $FeSO_4$; between pH 6 and 9, from the presence of $Ca(NO_3)_2$. To determine the effect of autoclaving upon strained tomato juice, ten flasks with 100 ml. each of solution 2 without agar were adjusted to pH values ranging from 3 to 9. All flasks were tightly stoppered with cotton plugs and then autoclaved for 15 minutes at 15 pounds. There were no appreciable changes in pH values of solutions with initial values of pH 3–6 (fig. 2, curve 2). Solutions initially above this range, however, experienced definite decreases in pH value upon autoclaving.

GERMINATION OF ORCHID SEEDS ON STERILE TOMATO-JUICE–AGAR MEDIA.—

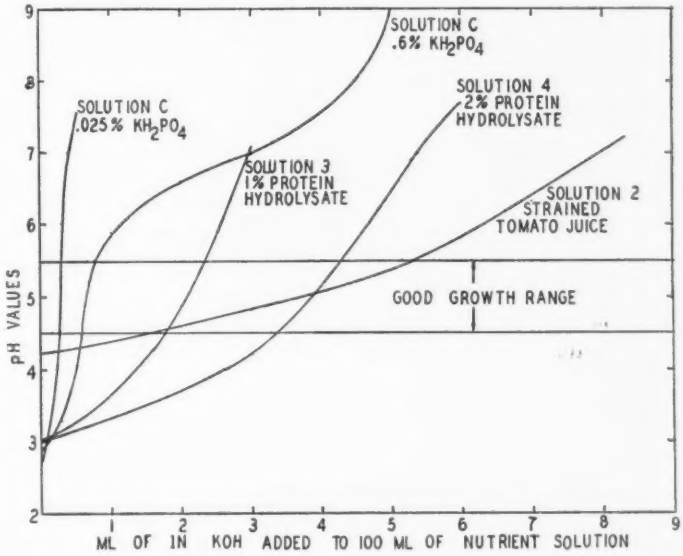

FIG. 1.—Buffer curves for solutions without agar, and maxium and minimum pH limits for satisfactory germination of orchid seeds and growth of seedlings.

Preliminary experiments in January, 1944, repeated later, indicated that orchid seeds would germinate on filtered tomato juice (solution 1) and strained tomato juice (solution 2) at a faster rate than on KNUDSON's solution B. Later, when KNUDSON (7) introduced his solution C, these experiments were repeated to compare the rate of germination on solutions 1 and 2 with that on solutions B and C. Each solution was tested in triplicate, 150 ml. of solution in a 500-ml. Erlenmeyer flask in each case. A

fourth flask in each series, with 100 ml. of solution, was used to check the pH value after autoclaving. All solutions were adjusted to pH 5.2 before the agar was added; after the addition they were adjusted by use of curve *2*, figure 2, so that after autoclaving the pH value would be 5.00. All flasks were tightly stoppered with hard cotton plugs and then autoclaved for 15 minutes at 15 pounds pressure.

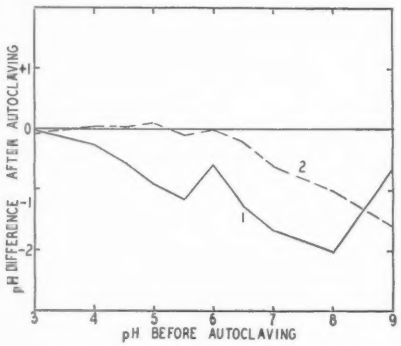

FIG. 2.—pH changes resulting from autoclaving (*1*) solution C and (*2*) strained tomato-juice solution 2.

In addition, to determine whether the inorganic or organic compounds of solution 2 were the effective growth factors, solution 2 was ashed to remove all organic compounds. In this procedure one volume of solution 2 was evaporated to dryness and then ashed until gray. The ash was dissolved in 25% HCl, and a sufficient amount of distilled water was added to attain the original volume before evaporation. This solution was added to like volume of double-strength solution B. Another ashed solution was prepared in a similar manner except that one volume of solution 2 was filtered and only the residue was ashed. As a control for the ashed solutions, a solution composed of one volume of double-strength solution B and one volume of solution 2 was used. After the addition of the required amount of agar, the cotton-stoppered flasks were autoclaved.

The first series of flasks with solutions 1, 2, B, and C were planted with seeds of *Epidendrum o'brienianum*. Later studies were made with hybrid *Cymbidium* seeds. The seeds were carefully removed from the seed pod in a sterile room so that sterilization of the seed just before planting was unnecessary. A microscopic examination of the *Epidendrum* seed showed that 83.9% had well-developed light-green embryos, 13.2% had incompletely developed embryos, and 2.9% seeds had no embryos. Just before planting, 1 ml. of sterile water was added to each flask, which was gently rotated to wet the entire agar surface. A sterile loop needle was used to plant the seeds, and the flasks were tilted so that the sterile water inside the flask could be used to wash the seeds off the planting needle. By this method the seeds can be spread quickly and evenly over the surface of the agar. The flasks were then placed in a special incubator with automatic temperature controls, night 22° C. and day 27° C. A small blower slowly moved the air to maintain uniform temperature throughout the incubator. Light intensity on cloudy days was approximately 150 foot-candles and on sunny days 500.

MEASUREMENT OF GROWTH RATE OF EMBRYOS.—As each embryo germinates, an approximately spherical protocorm is formed. Later a flat area develops from which the shoot emerges. KNUDSON (4) determined the growth rate by removing the embryos or protocorms from the flasks and measuring the diameter. This method is entirely satisfactory, since the protocorms are nearly spherical. In the present studies, however, measurement of the growth rate was desired without removing the plants from the sterile

flasks. For this purpose a special stage was constructed for a binocular microscope. The horizontal stage was raised at the near end of the scope, 45° from the horizontal, and a special fixture was added to hold the flask in position on the new stage, so that the relative diameters of the protocorms were always measured under the same observational conditions. The diameters were measured parallel to the flat surface of the protocorm. Since some of the protocorms elongate perpendicularly to the flat area, the volume based on the diameter of a sphere is a conservative measurement. Periodically the diameters of fifty protocorms selected at random from each series were measured. The average measurement of fifty protocorms inside the flasks was 1789μ and outside the flasks 1781μ.

RESULTS OF GERMINATION TESTS.—The results covering 150 days of growth from *Epidendrum* seeds are shown in figure 3. Both tomato-juice solutions 1 and 2 allowed significantly greater protocorm growth than did solutions B and C. In solutions B and C the stem and leaf growth was spindly, while in the tomato-juice solutions there was no stem or leaf growth. In the latter solutions the protocorms kept increasing in size, the cells continuing to enlarge and multiply with little or no differentiation. It was observed, as early as 1944, in cases in which the tomato-juice–agar medium shrank from the sides of the flask that the protocorms resting against the glass walls and not in direct contact with the medium began to differentiate, whereas those protocorms remaining on the medium did not differentiate but simply continued to increase in size. The protocorms on the glass walls produced sturdier seedlings than those on solutions B and C. These observations have been confirmed many times.

The results of the studies of seeds germinated on the ashed tomato-juice media are shown in figure 4. The ashed solutions (curves 3, 4) definitely were less effective than the control solution (curve 1). Little difference in size of protocorms was observed between those grown on solution 2 (curve 5) and on the combination of solution B plus solution 2 (control solution, curve 1). After 278 days of growth, however, the protocorms on the control solution began to develop leaves and roots, while those on solution 2 did not differentiate but only grew larger in size. When growth on the medium containing solution B plus solution 2 ashed was compared with that on solution B, no difference in size of protocorms was noted (curves 2 and 4, fig. 4); after 278 days, however, the protocorms on solution B developed leaves and roots at a faster rate.

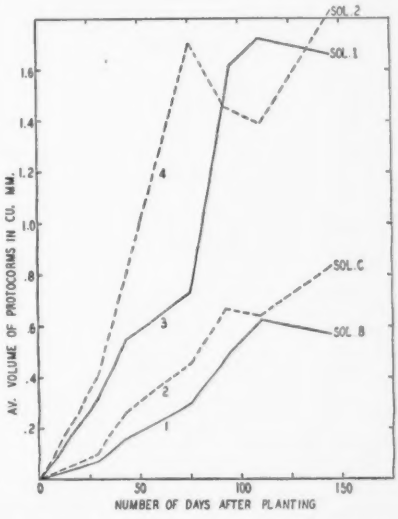

FIG. 3.—Rate of growth of embryos of *Epidendrum o'brienianum* on different media; 1, solution B; 2, solution C; 3, filtered tomato-juice solution 1; 4, strained tomato-juice solution 2.

The results of the studies with *Cym-*

bidium seeds on solutions 1, 2, B, and C are shown in figure 5. Not much difference was observed in the size of the protocorms grown on solutions 2, B, and C. There was a significant greater size, however, of protocorms grown on solution 1. Leaf growth on solutions B and C was excellent, whereas on solutions 1 and 2 the tiate, producing very sturdy seedlings that at the end of 2 months showed development equivalent to 1 year more than that of seedlings grown on solutions B and C.

Since the results of the experiments with ashed tomato juice indicated that organic compounds in the juice were re-

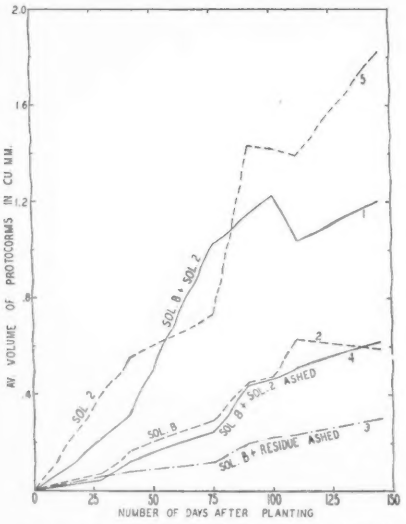

FIG. 4.—Rate of growth of embryos of *Epidendrum o'brienianum* on different media; *1*, solution B plus strained tomato-juice solution 2; *2*, solution B; *3*, solution B plus ash of residue filtered from solution 2; *4*, solution B plus ash of strained tomato-juice solution 2; and *5*, solution 2.

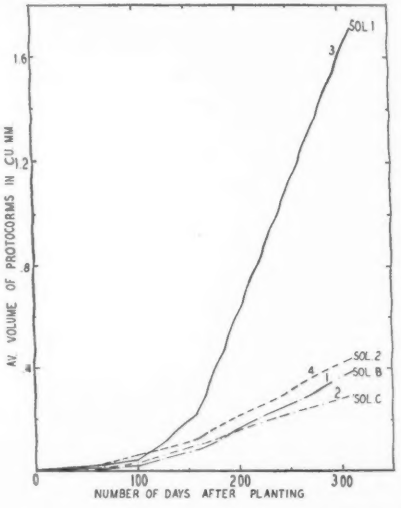

FIG. 5.—Rate of growth of embryos of hybrid of *Cymbidium* Hugh Evans and *Cymbidium* Flame in different media; *1*, solution B; *2*, solution C; *3*, filtered tomato-juice solution 1; and *4*, strained tomato-juice solution 2.

protocorms—as in *Epidendrum*—continued to increase in size but failed to differentiate. As in the case of the *Epidendrum* protocorms on solutions 1 and 2, however, those attached to the glass sides of the flasks and not resting on the agar did differentiate and produced a very vigorous leaf and root growth, far sturdier than that of the seedlings grown on solutions B and C. After 150 days the undifferentiated protocorms on solution 1 were removed from the flasks and placed on potted osmunda fiber. After 15 days in the greenhouse they began to differen-

sponsible for the faster growth of the protocorms on tomato-juice solutions than on solutions B and C, it was decided to add amino acids and vitamins to solution C. Two special solutions, 3 and 4, were prepared, using as a basis solution C to which a commercial brand of enzymatic protein hydrolysate, known as Prominogen, was added. Solutions 3 and 4 contained 1% and 2%, respectively, of this protein hydrolysate. Along with solution C and the tomato-juice solutions 1 and 2, they were used to study the germination of hybrid *Cymbidium* seeds.

Growth on solutions 3 and 4 was faster than on solutions 2 and C (fig. 6). Solution 4 was definitely toxic after 75 days, however, and after 200 days 95% of the protocorms had died. Solution 3 had a slightly toxic effect, and only 3% of the protocorms had died at 340 days. Once again, where the agar had separated from the glass walls, as in the case of solutions 1 and 2, the protocorms resting on the agar containing solution 3 did not differentiate, while those resting on the glass walls differentiated and grew vigorously.

Because of these general results, it seemed desirable to determine the buffering action of solutions 3 and 4 as compared with solutions 2 and C. Strained tomato juice (solution 2) had the strongest buffering action (fig. 1).

Discussion

It has been shown that certain orchid seeds germinate at a faster rate on dilute tomato juice supplemented with sucrose than on the previously used solutions (4–7) containing inorganic salts and sugar only. The tomato-juice media are better buffers than the media of KNUDSON in the pH range for good growth—pH 4.5–5.5 (fig. 1). During the growth period at least two dynamic factors are constantly acting to change the reaction of the culture medium: (*a*) the utilization of chemicals from the medium by the germinating embryos and (*b*) the products of growth. How effective the strong buffering action of the nutrient solutions containing organic compounds other than sugar may be, is now being investigated.

Preliminary experiments with KNUDSON's solution C buffered with 0.025% and 0.6% KH_2PO_4 indicate that the 0.6% solution is not so effective for growth as the 0.025% solution. The more strongly buffered solution C may precipitate out (9) more of the essential chemicals before they can be used by the germinating embryos. This dynamic factor and those mentioned above may be responsible for the abrupt changes in growth rate at approximately 100 days (fig. 3). All these dynamic factors must be considered in the preparation of a nutrient solution for optimum growth.

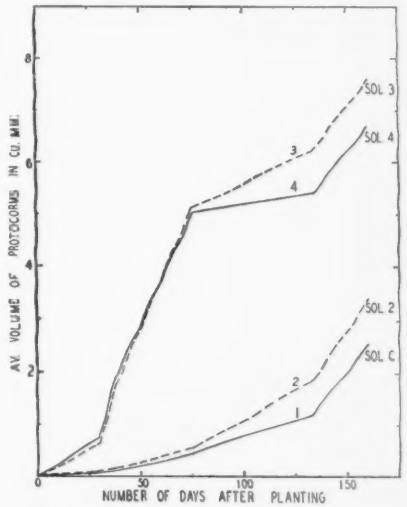

FIG. 6.—Rate of growth of embryos of hybrid of *Cymbidium* Shima Black and *Cymbidium* Flame in different media; *1*, solution C; *2*, strained tomato-juice solution 2; *3*, solution C plus 1% protein hydrolysate—solution 3; and *4*, solution C plus 2% protein hydrolysate—solution 4.

Upon autoclaving tomato-juice solution 2 no significant changes in pH value occurred within the pH range 3–6, in contrast to KNUDSON's solution C (fig. 2). Previous experiments (9) indicate that the change in pH value in solution C upon autoclaving may result from the precipitation of the $FeSO_4$ into insoluble iron salts. Since this precipitation may be increased in solution C strongly buffered with 0.6% KH_2PO_4, the observed decrease in the rate of germination and the apparent toxicity of this medium

might result from iron deficiency. If this is true, then the $FeSO_4$ used in solution C should be replaced by either organic or complex iron compounds that do not precipitate out in the presence of phosphate buffers. It is also suggested that autoclaving does not decrease the availability of iron compounds in tomato juice.

On the basis of growth results, one would be led to predict that tomato-juice media would be better than the inorganic solutions B and C for germinating orchid seeds and the growth of seedlings under aseptic conditions. That such is actually the case is shown in figures 3 and 6. Moreover, orchid seeds grown on inorganic solutions supplemented with ashed tomato-juice solutions in which the organic compounds were destroyed grew no faster than the controls grown without ashed tomato-juice supplement (fig. 4). It appears, therefore, that organic rather than inorganic constituents of the tomato-juice solutions are necessary for the enhanced growth.

This suggested that perhaps other natural organic matter added to an inorganic medium might be substituted for the tomato-juice media. When 1% of a commercial preparation of a protein hydrolysate, rich in amino acids and vitamins, was added to the inorganic medium and tested, the results (fig. 6) showed conclusively that orchid seedlings grown upon such a mixture develop much more rapidly than those grown on the inorganic-plus-sugar medium alone or on a tomato-juice solution. Work is now in progress to identify the components of the protein hydrolysate which are responsible for this greatly increased growth rate. It should, however, be noted that when the concentration of protein hydrolysate was increased from 1% to 2%, the more concentrated medium proved to be toxic.

It is noteworthy that, whereas the protocorms on the inorganic-plus-sugar media differentiated and at the end of 12 months the seedlings had developed sufficiently to be transplanted, the protocorms on either tomato-juice media or on those containing 1% protein hydrolysate failed to differentiate in almost every instance but simply increased in size. When these well-developed undifferentiated protocorms were transplanted from the flasks to suitable pots with osmunda fiber, however, they quickly differentiated, producing seedlings which were sturdier than those developed on inorganic-plus-sugar media. It is estimated that growing *Cymbidium* seedlings on the 1% protein-hydrolysate medium reduces the period of sterile culture some 6 months. Moreover, the seedlings which developed upon transplantation from this medium are a year advanced in development, or more, over the corresponding seedlings grown on inorganic-plus-sugar media. Also, because of the sturdiness of the former, their mortality rate upon transplantation is definitely reduced. All these seedlings will be grown to maturity, so that the type of plants developed can be described in detail.

In the few instances in which protocorms did differentiate when grown on tomato-juice media or on the 1% protein-hydrolysate medium, the circumstances seemed to be the same in each case—that the protocorms no longer rested on the agar medium but were attached to the wall of the flask. It thus appears that this condition is responsible, directly or indirectly, for the ensuing differentiation, but the causal factors are unknown. Whether the failure to differentiate in the other cases on tomato-juice media was due to physical or chemical influences cannot be definitely determined from the present available data. It is of interest that WHITE (13) pointed out that the use of such natural

plant tissue juices as xylem sap, phloem exudate, and liquid endosperm in the culture media for excised plant tissues has not met with much success. In many instances the natural juices were toxic. In 1941, VAN OVERBEEK *et al.* (10) successfully used coconut milk in a nutrient solution to grow *Datura* embryos. That differentiation can be controlled, however, by physical as well as by chemical factors has been shown by WHITE (12) in his work on oxygen gradients and by WENT (11) in his experiments on hormone gradients.

Summary

1. Germination and growth data indicate that tomato-juice–agar solutions are more effective than the synthetic culture media containing only sugar and salts used generally for the asymbiotic germination of orchid seeds. These tomato-juice solutions are more strongly buffered in the pH range, 4.5–5.5, for good growth, even when the concentration of KH_2PO_4 in the synthetic solutions is increased from 0.025% to 0.6%; likewise the pH values in the good growth range are not altered by autoclaving. Apparently the availability of the iron compounds in the tomato-juice solutions is not affected by either autoclaving or continued growth.

2. Ashing of the tomato-juice solutions definitely indicates that organic rather than inorganic compounds are responsible for the enhanced growth. A protein hydrolysate, rich in amino acids and vitamins, added to the inorganic solutions, allowed more rapid growth than even the tomato-juice media. Concentrations of protein hydrolysate greater than 1%, however, are toxic to *Cymbidium* embryos.

3. In general, on organic culture media the protocorms fail to differentiate, only increasing in size. As the agar dries out, thus leaving a space between the agar and the flask, those protocorms not resting on the media but on the walls of the flask do differentiate, producing seedlings sturdier than those on solutions B and C, and hence lowering the mortality rate upon transplantation. When the enlarged undifferentiated protocorms are transplanted to osmunda fiber, differentiation occurs, and in 2 weeks sturdy seedlings begin to develop.

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APPLICATION OF HERBICIDES TO CUT STUMPS OF A WOODY TROPICAL WEED[1]

JOHN G. TORREY AND KENNETH V. THIMANN

Introduction

The use of herbicides in the killing of woody weeds has been reported in a number of papers (**6, 12, 13, 14, 16**); standard spraying methods, essentially as in the treatment of herbaceous plants, were used. Shrubs and trees, however, present a number of special problems. STEWART and GAMMON (**11**) used a fog method of application for tall plants of wild grape. Also, the application of herbicides to the stumps of woody plants after cutting has sometimes been advocated, especially for the more resistant types. HAMNER and TUKEY (**6**) obtained apparent kills of a number of shrubs and young trees by applying 2,4-dichlorophenoxyacetic acid (2,4-D) as a salve to the cut surfaces of stumps.

The tropical weed *Dichrostachys nutans* Benth., referred to locally in Cuba as *aroma* or *marabú*, is a woody leguminous perennial. Its response to 2,4-D applied as a spray has already been reported on (**7, 13, 14**). The plant has remarkable powers of regeneration, ascribable in great part to horizontal underground roots which may extend for many feet, and from which may arise new ratoon growths. It has been found by EAMES (**4**) and is shown below that the roots are filled with stored starch, especially in the inner bark region and around the vessels of the xylem. Whereas individual clumps may be killed by the use of 2,4-D, the roots sometimes have been found to survive and establish new growth some months after the herbicidal treatment. In the further study of the problem of killing this woody plant, it was hoped that stump treatment would offer a new approach. The following report, therefore, deals with treatment of cut stumps and also with numerous experiments designed to throw some light on the entry and transport of the herbicide and the response of the plant.

[1] Publication No. 8, Journal Series, from the Atkins Garden and Research Laboratory of Harvard University, Soledad, Cienfuegos, Cuba.

Experimentation

TREATMENT OF CUT STUMPS

METHODS.—Areas of adult *aroma* plants, 4–6 feet in height, were marked out in plots approximately 20 × 22 feet (1/100th acre), and the plants were topped to the desired stump height with the use of pruning shears and machetes. Plots as nearly comparable as possible were used, averaging sixty to eighty stumps per plot with individual stumps of about 1–2 inches in diameter at the cut surface.

In the stump treatments emulsions were made in relatively high concentrations and painted on the cut surface of each stump, using a 1-inch paint brush. Approximately 30 ml. of emulsion were used per plot of sixty plants. In some cases application of the emulsion was made some time after the plants had been cut to stumps. In no case was this found to be critical so long as the time elapsed did not exceed 1 week. Painted applications were made between 9:00 and 11:00 A.M. in full sun, generally at about 92° F. (air temperature), so that treatment was followed usually by 6 hours or more of full sun. Grass growing between stumps was cut back periodi-

cally so as to leave the stumps clear and unshaded. Spray applications to the leaves of newly arising basal shoots ("ratoons") were made with a 4-gallon knapsack sprayer, at a rate of about 20 gallons of emulsion per acre.

Readings were made every 2 weeks for a period of 3 months. The following categories were established as a means of expressing the degree of effective treatment over extended periods of time. The number of plants in each category was expressed as the percentage of the total number, established by actual count in every case.

% *Old Green:* Percentage of total number of plants which retain any green foliage which was present on foliar branches at the time of initial treatment. This corresponds to the sum of the classifications "apparently intact" and "somewhat damaged" of THIMANN (**14**).

% *New Green:* Percentage of total number of plants which, following defoliation, showed new young green shoots or leaves.

% *No Green:* Percentage of total number of plants which showed no foliage whatsoever. This corresponds to the previous classifications "apparently killed" and "seriously injured or dying" (**14**).

CHOICE OF HERBICIDE.—Earlier work had established that 2,4-D in various forms is an effective agent when applied as a spray to the leaves of the adult *aroma* plant or young ratoons. Subsequent extensive comparisons of the different forms established that the esters were definitely more effective than the salts (**7**). In the present experiments on stump treatment the butyl ester of 2,4-D was used, being applied in varying concentrations as noted below. For comparison, the butyl ester of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was utilized in a series of concentrations. Sodium arsenite, reported by GRAY (**5**) as an effective herbicide against morning glory and later shown to be effective against deep-rooted perennial weeds by CRAFTS (**2**), was also tested in stump treatments. It was ineffective against *aroma* as a spray, causing rapid defoliation followed by vigorous growth of apparently normal shoots. Gas oil, applied to the ringed trunk bases, has been utilized as a local expedient in Cuba in an attempt to control *aroma*. Gas oil also has recently been found effective in emulsion mixtures with 2,4-D in the spray treatment of certain tropical weeds (**3**). Gas oil was therefore tested alone and with 2,4-D as a herbicide, using the stump treatment.

The results of a series of tests of different preparations applied to the stumps of *aroma* are shown in figure 1. The percentages of the total number of plants showing No Green were recorded over a period of weeks, beginning after the second treatment. The rapid appearance of new foliage within 4 weeks after the arsenite treatment (38% solution) is very striking. More dilute solutions were, as expected, even less effective. An aqueous emulsion of the butyl ester of 2,4-D at optimum concentration was established as the most effective herbicide, surpassing all others tried.

It will be noted that development of new shoots was definitely more delayed in the "short" stumps, cut almost to ground level, than in the stumps 2 feet long. Although quite to be expected, this point is of practical value in treatments of this sort.

EFFECT OF CONCENTRATION.—Since only very small volumes can be applied to the cut surface, it was evident that high concentrations would be necessary. Mixtures containing 10, 20, 30, and 40% of the butyl esters of 2,4-D and 2,4,5-T in aqueous emulsion were therefore made up. In each case, two treatments were made 1 week apart. The value of the second treatment is established below. From the data (table 1) it is clear that the 20%

concentration of the butyl ester of 2,4-D is the optimum for this herbicide. 2,4,5-T was consistently less effective than the same concentration of 2,4-D; in the readings it is evident that after 8–9 weeks the extent of new growth was very much greater from the plants treated with 2,4,5-T.

mixtures were made utilizing gas oil instead of water as the diluent, and the stumps were treated with these mixtures in the same way as with the aqueous preparations. The oil used was a light diesel oil (Standard Oil of Cuba), viscosity (100° F.) 35–45, flash-point minimum 150. The results (table 3) indicate

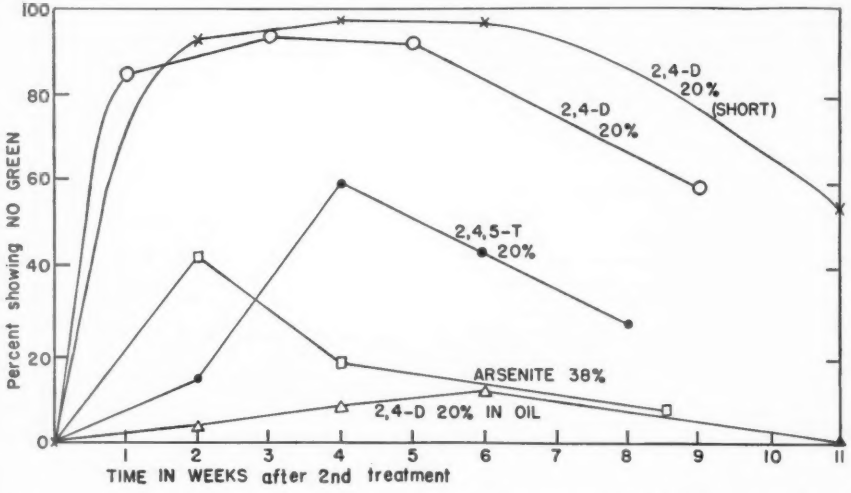

FIG. 1.—Stump treatments of *aroma marabú*. Each application was made with brush on cut surface and repeated 2 weeks later. Number of plants counted to determine each point varied from 50 to 200. All stumps were about 2 feet high except those marked "short," which were only 2–3 inches above ground level. The legends *2,4-D* and *2,4,5-T* refer to butyl esters of these acids, which were in aqueous emulsion except for one series in oil.

EFFECT OF REPEATED TREATMENT.—To determine whether one treatment is sufficient, half of each plot in one experiment was treated a second time 1 week after the initial treatment, using the same concentrations and means of application. The results of such experiments with 2,4-D ester emulsions (table 2) indicate that at the lower concentrations two treatments are considerably more effective than one. With 30 and 40% emulsions, however, the second treatment makes little difference.

EFFECT OF GAS OIL IN APPLICATION MIXTURE.—Using the 2,4-D butyl ester, that oil markedly retards the rate of action of 2,4-D. The oil apparently blocks the entrance of the ester into the stump, allowing only local action. This blocking effect was manifested macroscopically by a ring of callus tissue in the circumference of each stump treated with the oil—2,4-D mixture at 1–2 inches below the surface of application. Above this callus ring the trunk was dry and discolored; below the ring and in the callus itself the trunk was green and living (fig. 2*C*). A similar callus was noted in many of the stumps treated with the aqueous emulsion of 2,4,5-T ester (fig.

2*B*), although of less extent. In no case was such callus formation observed in the control plants or in those treated with the aqueous emulsions of 2,4-D (fig. 2*A*). MINSHALL and HELSON (**8**) and ROHRBAUGH (**10**) found that diesel oil, applied to leaves, entered the intercellular spaces of the plant tissue and not the cells themselves. It is possible that distribution of the oil in a similar manner from the cut surface of the stump blocks the transport of 2,4-D, by preventing the normal oxidative processes on which transport probably depends. In any event, sprouting took place vigorously from the lower parts of the stumps treated in this way, as shown by the data plotted in figure 1.[2]

It seems possible that this relative ineffectiveness of oil mixtures is a phenomenon peculiar to the plant studied, since results recently reported by BEATTY (**1**) indicate satisfactory killing of northern trees with similar treatments.

TABLE 1

COMPARISON OF HERBICIDAL EFFECTS OF BUTYL ESTERS OF DI- AND TRI-CHLOROPHENOXYACETIC ACID IN AQUEOUS EMULSION, AND EFFECT OF CONCENTRATION. ALL PLANTS GIVEN TWO TREATMENTS ON STUMPS. RESULTS EXPRESSED AS "% NO GREEN" (SEE TEXT)

PER CENT	WEEKS AFTER SECOND TREATMENT		
	3	5	9
Concentration of 2,4-D:			
10	42.0	60.3	40.3
20	93.8	91.0	55.9
30	77.4	64.8	47.5
40	71.5	65.6	30.2
	WEEKS AFTER SECOND TREATMENT		
	4	6	8
Concentration of 2,4,5-T:			
10	27.9	20.9	10.9
20	59.3	45.0	27.6
40	86.9	51.8	25.7

ENTRANCE OF 2,4-D INTO PLANT

In order to shed light on the ability of buds to develop from the base or roots of plants whose tops were "apparently killed," an attempt was made to study the penetration of the herbicide into the plant. For this purpose use was made of the slit pea-stem test (**15, 17**) and of the cucumber-root inhibition test (**9**). For the extraction, representative plants

[2] In later observations, development of shoots on the oil-treated plants was less active than after treatment with aqueous emulsion, so that after 8 months there was little difference between oil and water. Oil treatment, however, showed no advantage. With 10% 2,4-D the "% No Green" after 8 months was: in water 13, in oil 14; with 20% 2,4-D: in water 27, in oil 30.

TABLE 2

COMPARISON OF SINGLE AND DOUBLE TREATMENT OF STUMPS EXPRESSED AS "% NO GREEN"

	10% 2,4-D		20% 2,4-D		30% 2,4-D		40% 2,4-D	
	Weeks after second treatment							
	5	9	5	9	5	9	5	9
One treatment	49.1	40.6	60.6	29.0	64.7	45.6	58.0	32.5
Two treatments (1 week apart)	60.3	40.3	91.0	55.9	64.8	47.5	65.6	30.2

were selected, and the desired tissue was cut into small pieces, including all parts of the particular plant structure to be analyzed. Five-gm. samples were extracted at room temperature for 12 hours, using 25 ml. of ethyl ether freshly distilled over $FeSO_4$. The ether extract was poured off and the tissue rinsed with 5 ml. of fresh ether. This crude extract, when evaporated down and tested, without further purification, in the cucumber-root inhibition test, was found to inhibit root elongation markedly, whether the plant extracted had been treated with 2,4-D or not. For this reason all subsequent extracts were purified in the following way. To the ether extract was added half its volume of 1% $NaHCO_3$, and the mixture shaken gently. The ether was discarded, and the carbonate solution was acidified with 1 *N* HCl to about pH 2. Then the acidified aqueous solution was extracted twice with equal volumes of ether, the ether evaporated down, and the residue taken up in 10 ml. of water. From this was made a logarithmic dilution series. This purification procedure removed the inhibiting effect of extracts from control plants. The effect is doubtless due to the nonacidic growth-inhibiting substances often reported in plant extracts.

TABLE 3

EFFECT OF BUTYL ESTER OF 2,4-D IN WATER AND IN GAS OIL; FIVE WEEKS AFTER SECOND TREATMENT, EXPRESSED AS "% NO GREEN" (SEE TEXT)

% 2,4-D	PER CENT	
	In water	In gas oil
10	60.3	8.6
20	91.0	15.2

In the slit pea-stem test, stem sections, cut from the second to third nodes of 8-day-old Alaska pea seedlings grown in wet sand in the dark at about 20° C., were slit longitudinally for 3 cm. at the apical end, washed for 1 hour in water, then placed, six sections per dish, in Petri dishes containing 20 ml. of solution. After 24 hours of continued darkness the curvatures were measured with a protractor, and the average angle of curvature determined for each solution. Satisfactory concentration curves were readily established with known concentrations of 2,4-D butyl ester. By comparing these concentration curves with curves determined from the extracts, the probable 2,4-D content of the tissue was de-

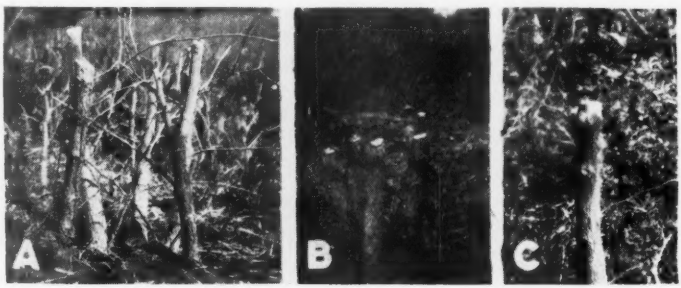

FIG. 2.—Long stumps after two applications of herbicide to cut surface. *A*, 20% 2,4-D aqueous emulsion; 10 weeks after second treatment. No swelling. *B*, 20% 2,4,5-T aqueous emulsion; 7 weeks after second treatment. Swollen ring 3–5 cm. below cut surface. *C*, 20% 2,4-D emulsion in oil; 6 weeks after second treatment. Marked swollen ring 3–5 cm. below cut surface.

duced. The results of these extraction experiments (table 4) are expressed as concentration in molarity of 2,4-D, deduced from concentration curves. Repetition of the experiments showed essentially the same results, namely, that no 2,4-D whatever could be found in the roots, although the test was easily capable of detecting minute amounts.

Because of the difficulty in growing uniform pea seedlings under hot tropical conditions, a parallel series of extracts was tested for cucumber-root inhibition.

Results (table 4) confirm the evidence obtained in the pea test. With one possible exception, there was no evidence of 2,4-D in any of the root extracts, whether the plants had been treated only as short a time as 4 days, when defoliation was imminent, or over longer periods up to $2\frac{1}{2}$ weeks when defoliation was complete. Evidence points to the entrance of the growth substance into the ratoon stems and trunk but not into the root itself. In the one exception the apparent concentration was on the border line of sensitivity

TABLE 4

DEDUCED CONCENTRATIONS OF 2,4-D IN TISSUES EXTRACTED AFTER 2,4-D TREATMENTS

MATERIAL EXTRACTED	DAYS AFTER CUTTING	DAYS AFTER 2,4-D ESTER SPRAY AT 1.0%	DEDUCED CONCENTRATION (IN MOLS)	
			Pea test	Cucumber test
Root of topped stump	27	17	0	.....
Root of topped stump	27	13	.....	0
Root of topped stump	26	Untreated	0	0
Root of topped stump	53	6	0	.....
Root of ratoon of sprayed plant	Uncut	6	0	.....
Root of ratoon of sprayed plant	Uncut	4	.....	(0.1×10^{-5})
Trunk of ratoon of sprayed plant	Uncut	6	2.0×10^{-5}	.....
Trunk of ratoon of sprayed plant	Uncut	9	.....	13×10^{-5}
Stems of ratoon of sprayed plant	Uncut	9	.....	45×10^{-5}

The test is reportedly sensitive to 2,4-D in about the same range as the pea test, namely, 5.0–0.005 p.p.m. (**9**). Extractions were made as described above, and serial dilutions using 15 ml. per Petri dish were prepared. Fifteen cucumber seeds (variety Early Fortune)[3] were placed on filter paper in each dish, and the length of the primary root was measured at the end of 96 hours' germination in the dark at 29° C. Root lengths obtained in the unknown extracts were compared with those in known concentrations of the 2,4-D butyl ester, and the concentration of 2,4-D in the unknown deduced therefrom.

[3] Kindly supplied by Dr. A. G. NORMAN, to whom we wish to express our thanks.

of the test under the conditions prevailing. Data on extracts of stems and trunks recorded in table 4 did not wholly exclude surface residues of the herbicide and should not necessarily be interpreted as showing internal concentration alone. It should be added that the fact that this test depends on an inhibition and not on a growth promotion makes it possible that the apparent activity was not solely due to 2,4-D in spite of the preliminary purification of the extracts.

EFFECTS OF 2,4-D TREATMENT ON STARCH CONTENT

It had been noted that the roots of *aroma* were filled with starch, which could be demonstrated readily by stain-

ing fresh sections with an aqueous solution of 1% iodine in 1% KI. Using 400 × magnification, numerical counts were made of the grains in an entire field, including representative areas either in the phloem or bark region or in the xylem.

Starch counts in the roots of 2,4-D-treated ratoons (table 5) show clearly that the starch content was markedly reduced following treatment with 2,4-D spray and that this reduction was correlated closely with the onset of defoliation.

TABLE 5

STARCH COUNTS IN ROOT TISSUE OF RATOONS OF PLANTS SPRAYED WITH 1.0% 2,4-D

No. of days after spraying	Appearance of plant	No. of starch granules per microscopic field (400×)	
		Xylem	Phloem
0	Foliage green	384	*ca* 1000
3	Foliage yellowing	278	700
4	Foliage browning	304	105
6	Defoliated	214	68
6	Defoliated	103 *	46 †
6	Defoliated	154	108

* Mean 157. † Mean 74.

TABLE 6

STARCH COUNTS IN ROOT TISSUE OF 2,4-D-TREATED AND UNTREATED STUMPS OF "AROMA MARABÚ"

Root no.	2,4-D treatment	No. of days		Final appearance of plant	No. of granules per microscopic field (400×)	
		After cutting	After 2,4-D treatment		Xylem	Phloem
1	None	36	.....	1–2-ft. ratoons	85	0
2	None	69	.....	2–3-ft. ratoons	184	352
3	1% spray	47	10	2-ft. ratoons, defoliated	65	3
4	0.5% spray	24	11	1–2-ft. ratoons, defoliated	61	0
5	20% painted twice	50	50	No ratoons	214	632
6	20% painted twice	71	71	No ratoons	138	195

Roots to be tested were dug up and hand-sectioned with a razor blade, stained for 10 seconds in the iodine solution, washed in distilled water, and mounted under a glass cover slip. By use of this technique, observations were made periodically on the roots of treated plants in order to determine the effect, if any, of 2,4-D treatment on the starch content. Both intact and topped plants were observed.

The effect on starch counts of cutting adult plants to stumps and then treating subsequently with 2,4-D is shown in table 6. It is apparent that the removal of foliar tissue which results from topping, and which is followed by ratooning from the base or from main roots near the base, causes at first a reduction in the starch content of the root (root no. 1). Later, when the ratoons provide photo-

synthetic materials above the immediate needs of the plants, these are stored in the roots as starch (root no. 2). Spraying the young ratoons with 2,4-D acts together with the stump-cutting to maintain a very low starch content in the roots (roots nos. 3 and 4) as a result of removing the source of new carbohydrate. The counts in these roots are much lower than in those of table 5. It is to be noted that starch deposits in the xylem are considerably more stable than in the phloem and show a lag in every case compared with the starch content of the phloem in the same root. This indicates that, as would be expected, the starch in the phloem is much more directly dependent on supply from the leaves than that in the xylem. Painting the stumps with 2,4-D solutions prevents the formation of new ratoons by a prolonged bud inhibition, but over this long period there is apparently a replenishment of the storage starch in the root (nos. 5 and 6). In view of the very extensive root systems of these plants, it seems likely that carbohydrate may be supplied from interconnected, untreated plants at a distance. This may, of course, likewise occur without bud inhibition, as in root no. 2. It might be predicted that plants showing low starch content after such a prolonged period as 70 days will show little or no ratooning, whereas high-starch content would indicate probable regrowth. Removal of starch from the roots of *aroma* plants may indeed be a prerequisite to ultimate killing.

Conclusions

The data of these experiments concerning the transport of 2,4-D and its effect on the plant point to the following conclusions. There is no evidence that 2,4-D, applied either as a spray or in paint treatments, enters into the roots of the *aroma* plant (within 17 days) in concentrations demonstrable by the tests utilized, although it does penetrate into the trunks. 2,4-D solutions, when applied to the above-ground parts of the plants, act to reduce the carbohydrate stores present in the roots in the form of starch. Starch content of the roots reflects the condition of the plant and can be lowered by repeated defoliation, either as a result of herbicidal treatment or by topping. It can apparently also be replenished, perhaps from plants with interconnected roots at some distance away. The early stages of regrowth cause a reduction in starch content, and spraying at the appropriate time after growth has begun can therefore be very valuable in bringing about an exhaustion of the plant's reserves. It appears essential to treat all plants in one area at the same time, so as to allow no source for replenishment of carbohydrate to the plants. Topping and 2,4-D treatment act in the same direction toward the removal of storage starch in the roots.

Summary

1. The effectiveness of several herbicides when applied in high concentration by painting on the cut stumps of *aroma* or *marabú* (*Dichrostachys nutans*) has been determined.

2. A 20% aqueous emulsion of the butyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D), applied twice, was the most effective of the preparations tried in repressing new shoot growth. Higher or lower concentrations of this emulsion, the butyl ester of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and concentrated sodium arsenite solutions were all less effective.

3. The addition of gas oil to the 2,4-D ester greatly retarded the latter's ef-

fectiveness, and there was evidence of inhibition of its transport in the stump.

4. Extraction and bio-assay of the 2,4-D in portions of treated plants showed no evidence of its entry into the roots, within $2\frac{1}{2}$ weeks, though it was detected in stems.

5. Removal of the tops of the plants causes a decrease in starch content of the roots, but over a period of several weeks the starch content may rise again, possibly as a result of contributions from other plants on the same root system.

6. Defoliation by treatment with 2,4-D also brings about great reduction in starch content, but unhindered development of leafy shoots increases it. It is suggested, therefore, that repeated defoliation may be necessary for killing the underground parts of the plant.

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STUDIES IN CROP PHYSIOLOGY: CELL-SIZE CHARACTERISTICS OF SUGAR-CANE VARIETIES IN RELATION TO DROUGHT RESISTANCE

K. N. LAL AND O. N. MEHROTRA

Introduction

Ever since the classical researches of ZALENSKI (61–63), KOLKUNOV (20–24), YAPP (59), RIPPEL (38), and RÜBEL (39), considerable emphasis has been laid on the relationship between the anatomical characteristics of leaves and the capacity of plants to resist drought. During recent years analysis of this major problem of drought resistance in relation to cell size has progressed along certain specified lines. Although some investigators have laid emphasis on the stomatal characteristics of the species as of vital importance in such analysis (7, 9, 15, 28, 40, 42, 48, 49), others have considered the role of environment on the general development and anatomical features to be equally important (12, 13, 19, 30, 33, 36, 40, 47, 54, 55, 57, 59, 62). The third line of interest concerns the relation which the age and development of the plant bears to the cell-size characteristics of different species (30, 37, 59). Researches conducted in these directions (5, 10, 18, 25, 40, 50, 52, 53, 56, 60) indicate that the upper leaves of a plant are more xeromorphic in structure than the lower ones and that high stomatal frequencies are usually associated with more xerophytic conditions.

Cell size is also suggested to control several physiological processes such as water relations (6, 11, 44, 45, 51), development of suction pressure (32), and exhibition of sleep movement (53). Hardening for drought (8, 16) or frost resistance (2, 34) is also known specifically to bring about an increase in size of the water-conducting cells and in the number of stomata. Equally interesting is the relationship between the stomatal characteristics and (*a*) phylogenetic classification of plants (35) and (*b*) chromosomal characters of different species (3, 10, 14, 17, 31, 43, 46, 48, 49).

These investigations thus emphasize the specific relation that cell size bears to certain physiological responses and behavior of plants. In sugar cane, so far as our information goes, no systematic attempt has been made to explain this problem. The only outstanding contribution on this subject has been made by MAMELI-CALVINO (27), who pointed out that stomata in sugar cane are more numerous on the lower surface, are rather sunken, and are always protected by wax and often by hair. Leaves have limited intercellular space, and water-conducting tissues are well developed. Equally interesting are the observations of ARTSCHWAGER (1) on number and size of epidermal cell types in various species of *Saccharum*.

An attempt is therefore made in this paper to evaluate the significance of cell characteristics in the major problem of growth and development of this economic crop. The inquiry has been extended to elucidate the effect of such factors as (*a*) varietal behavior, (*b*) age and development of leaves and leaf surface, (*c*) stage of tiller and plant development, and (*d*) chromosome number. Indications have also been given to illustrate how far such a study would be helpful in the classification of sugar-cane varieties

into drought-resistant, medium drought-resistant, and drought-susceptible forms. The relationship between growth performance and size of stomatal and epidermal cells will be discussed in a later communication.

Material and methods

Experiment I

Selection of varieties.—Twelve sugar-cane varieties, consisting of standard Coimbatore and indigenous strains growing on the college farm under one condition of soil nutrition, were selected. These included Co 453, 205, 385, 421, 312, 313, 356, 213, 299, 290, Co S 76, and Rheora. All observations were recorded in December, 1946, when the crop was more or less mature for harvesting (age 240 days).

Sampling technique and preparation of slides.—Since environmental conditions and the position of the leaf on the plant have been found to affect the size and frequency of stomata (**10, 13, 40**), the plants were grown under similar cultural conditions, and counts and measurement of cells were made on fully expanded healthy leaves from the primary shoots. After detaching, the leaves were brought to the laboratory with their basal part dipped in water and kept overnight in this position for study on the following day. Preliminary observations indicated that there was no appreciable difference in cell size and frequency from different peelings, which were, therefore, taken from the portion midway between the apex and base and the midrib and margin (cf. **10, 46**).

Leaves of sugar cane are exceptionally stiff, and it is difficult to strip off the epidermis for microscopic studies of cell size. The procedure adopted, therefore, consisted of taking small pieces of leaf blade and scraping off the tissues other than those of the epidermal regions. This was done for both the upper and the lower surfaces of leaves by means of a fine razor blade. Lloyd's (**26**) techniques for preparation of slides and for cell-size measurement were employed. The peeled epidermis obtained after scraping was fixed in absolute alcohol; it was then mounted in a drop of glycerine, and the cover slip was sealed by means of zinc cement. This enabled the slides to be examined at convenience.

Measurement of cells and counting.—Measurements of the size of stomatal and epidermal cells of both surfaces of each leaf were made with an eyepiece micrometer, a Zeiss microscope with a 15× ocular and 40× objective being used; cell counts were made with a reticulated 7× eyepiece and an 8× objective. The total number of stomata occurring per four small squares of the ocular micrometer (0.01 sq. mm.) were determined at five different places on the strip in the interveinal regions. The data were recalculated to number of cells per square millimeter and are so expressed in the tables.

Stomatal index.—Stomatal index was calculated by Salisbury's formula $I = S/S + E \times 100$, where I stands for stomatal index, S is the number of stomata per unit surface, and E is the number of epidermal cells for the same unit area.

Statistical analysis.—The data were analyzed by the analysis-of-variance method. The variance due to varieties, surface, samples, and interactions between variety and surface were calculated separately in order to estimate the error variance after the manner indicated in table 1.

Experiment II

The second part of the investigation consisted in selecting only two of the

varieties, Co 205 and Co 421. Epidermal peelings were taken from leaves of four stages of development, and such leaves were selected from youngest and oldest tillers. Including the two surfaces of leaf, therefore, the factors involved were: varieties, 2; surfaces, 2; tiller age, 2; and leaf age, 4; thus making a total of 32. Analysis of variance was used to assess the significance of the results; the procedure is given in table 2.

Summarized results of these two investigations are presented in the tables, each dealing with one of the following characters: length of epidermal cells, breadth of epidermal cells, number of epidermal cells, length of stomata, breadth of stomata, number of stomata, and stomatal index. In all these cases the standard error and critical difference have been given to indicate the significance of various pairs of comparisons.

TABLE 1

CELL CHARACTERS OF SUGAR-CANE VARIETIES (EXPERIMENT I). ANALYSIS OF VARIANCE

Owing to—	D.F.	Mean sum of squares for different characters						
		Epidermal cell			Stomata			Stomatal index
		Length	Breadth	Number	Length	Breadth	Number	
Replication	4	4102	5.7	29083	23.7	5.7	28208	34.5
Varieties	11	1047	11.6	155363	61.9	49.9	28568	70.5
Surfaces	1	1042	31.8	675000	93.6	0.5	2914083	2622.5
Var.×sur.	11	950	17.48	64090	124.8	18.06	41901	71.5
Error	92	271	3.84	19213.7	4.2	2.3	14034	15.3
Total	119							
S.E./Obs.		13.8	1.9	138.6	2.04	1.05	118.4	3.9

TABLE 2

CELL CHARACTERS OF SUGAR-CANE VARIETIES (EXPERIMENT II). ANALYSIS OF VARIANCE

Owing to—	D.F.	Mean sum of squares for different characters						
		Epidermal cell			Stomata			Stomatal index
		Length	Breadth	Number	Length	Breadth	Number	
Replication	4	182	8.8	8281	5.7	3.7	11343	22.3
Varieties	1	0	7.6	90250	1071	22.5	3062	27.4
Tiller age	1	265	12.5	9000	1.0	1.6	232562	283
Surface	1	129	54.0	841000	22.0	46.2	4000562	4328
Leaf age	3	114	18.2	48916	91.6	42.2	2895	18.5
Var.×tiller age	1	448	35.8	36000	34.0	244.4	45562	8.2
Var.×surface	1	105	0.2	169000	26.0	3.0	22562	174.4
Var.×leaf age	3	62.2	5.2	42916	8.3	23.2	33562	11.6
Sur.×tiller age	1	18.2	49.8	132250	8.0	34.2	203062	78.3
Sur.×leaf age	3	226.4	3.8	68666	31.6	2.6	56062	49.6
Higher order interactions	16	187.2	7.5	53921	37.2	2.3	45562	39.6
Error	124	103.7	0.12	106.36	2.5	1.0	2069.5	14.7
Total	159							
S.E./Obs.		10.1	0.34	103.1	1.5	1.0	45.4	3.8

Results

Characteristics of epidermal cells

Length.—The length of epidermal cells varied significantly in response to varieties and surfaces (table 1); both the main effects due to these factors were highly significant. Interaction between variety and surface also exhibited significant effects. The lower surface of the leaf showed significantly shorter lengths of epidermal cells (table 3) than those of the upper. Of all the varieties, Co 421 exhibited the maximum epidermal cell size, followed by Co 299, 453, and 312. Three classes could distinctly be noticed: class I, exhibiting high epidermal cell length, ranging from 82.54 to 99.36 μ and comprising varieties Co 312, 453, 299, and 421; class II, showing a medium range of 71.55 to 79.65 μ and comprising Co 356, 213, Co S 76, Rheora, and Co 290; and class III, consisting of varieties Co 385, 313, and 205, having the epidermal cell length between 63.45 and 68.50 μ. As a class varieties belonging to the first category exhibited significantly greater epidermal cell length than varieties of the third group. The differences between varieties of classes I and II or between those of classes II and III were, however, not significant in all the pairs of varietal comparisons.

Experiments conducted toward the end of the life-cycle further indicate the main effects of tiller age to be highly significant (table 2). Interaction due to variety × tiller age and surface × leaf age were also significant. No significant difference in the length of epidermal cells on older and younger tillers was noted (table 4). Differences due to surface, variety, and leaf age were also insignificant.

TABLE 3

Surface and variety effects upon length of epidermal cell (experiment I)

Varieties	Surface (mean of 5 values)		Mean of 10 values	Difference
	Lower	Upper		
Co 299	81.54	93.26	87.40	
Co 213	71.28	72.90	72.09	15.31
Co 290	81.54	77.76	79.65	7.56
Co 421	95.58	103.14	99.36	19.71
Co S 76	59.44	91.26	75.60	23.76
Co 453	70.74	96.12	83.43	7.83
Co 312	80.46	84.62	82.54	0.89
Rheora	63.72	91.10	77.41	5.13
Co 356	64.26	78.84	71.55	5.86
Co 385	72.90	54.00	63.45	8.10
Co 205	63.02	73.98	68.50	5.05
Co 313	82.46	46.3	64.38	4.12
Mean	73.9	80.2	C.D. for varieties = ± 11.64	
Difference	6.30		C.D. for surfaces = ± 4.76	

Breadth.—On the breadth of epidermal cells, variety and surface main effects and variety × surface interaction were significant (table 1). Breadth of epidermal cells on the undersurface was significantly lower than on the upper (table 5). Co 421 showed the maximum epidermal cell width which was significantly greater than that in Rheora; the

latter was significantly inferior to all the other varieties as regards this character. Except for these two varieties—Co 421 with maximum breadth and Rheora with minimum epidermal cell width—others showed an average cell breadth between 14.58 and 16.74 μ. The varieties could be classified as follows: class I, in which the cell width ranged between 16.74 and 17.28 μ, comprising Co 312 and 421; class II, having a range of 14.85–16.20 μ with varieties Co 385, 313, 290, Co S 76, Co 299, 213, 356, and 453; and class III, with Rheora and Co 205 having a breadth of 12.96–14.58 μ. Varieties of class I differed significantly from varieties belonging to class III. The differences among individual varieties belonging to each of the above classes were, however, not significant.

In the second experiment the breadth of the epidermal cells was also found to vary significantly in response to all the main effects—variety, tiller age, surface,

TABLE 4*

TILLER AGE, SURFACE, VARIETY, AND LEAF AGE EFFECTS UPON LENGTH OF EPIDERMAL CELLS (EXPERIMENT II)

Leaf age	Tiller age		Surface		Variety		Mean
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	98.65	97.50	93.85	102.30	97.80	98.35	98.07
II	97.40	90.50	94.40	93.50	92.85	95.05	93.95
III	95.45	96.0	96.85	94.60	95.35	96.10	95.72
IV	97.05	94.25	94.70	96.60	97.45	93.85	95.65
Mean	97.13	94.56	94.95	96.75	95.86	95.83	.....

* C.D. at 5% for variety, tiller age, and surface = ± 3.08; for leaf age = ± 4.48.

TABLE 5

VARIETY AND SURFACE EFFECTS UPON BREADTH OF EPIDERMAL CELLS (EXPERIMENT I)

Variety	Surface		Mean	Difference
	Lower	Upper		
Co 299	14.04	16.74	15.39	
Co 213	17.28	15.12	16.20	0.81
Co 290	13.50	17.26	15.38	0.82
Co 421	16.74	17.82	17.28	1.90
Co S 76	15.66	15.12	15.39	1.89
Co 453	15.12	17.28	16.20	0.81
Co 312	15.66	17.82	16.74	0.54
Rheora	10.26	15.66	12.96	3.78
Co 356	17.28	15.12	16.20	3.24
Co 385	13.50	16.20	14.85	1.35
Co 205	16.20	12.96	14.58	0.27
Co 313	14.58	15.66	15.12	0.54
Mean	14.98	16.06	C.D. for varieties = ± 1.68	
Difference	1.08		C.D. for surface = ± 0.67	

and leaf age (table 2). First-order interactions, due to variety × tiller age, variety × leaf age, surface × tiller age, and surface × leaf age, and higher-order interactions were equally significant. Significant differences between the oldest and the youngest tiller, between the lower and the upper surface, and between the two varieties Co 421 and Co 205 were also recorded (table 6). The youngest leaf showed significantly greater breadth than the second and fourth, while the third showed significant differences from both the second and the fourth leaf in the chronological order of development.

NUMBER.—On the number of epidermal cells, main effects due to variety and surface and interaction due to variety and surface were all highly significant (table 1). The lower surface of the leaf on an average exhibited a larger number of epidermal cells per unit area than the upper (table 7). The varieties

TABLE 6*

TILLER AGE, SURFACE, VARIETY, AND LEAF AGE EFFECTS ON BREADTH OF EPIDERMAL CELLS (EXPERIMENT II)

LEAF AGE	TILLER AGE		SURFACE		VARIETY		MEAN
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	15.0	15.5	14.5	16.0	15.30	15.20	15.25
II	15.0	14.7	14.2	15.5	14.8	14.9	14.85
III	16.75	14.5	15.5	15.7	15.75	15.56	15.62
IV	14.25	13.85	13.2	14.8	14.8	13.30	14.05
Mean	15.25	14.63	14.36	15.52	15.16	14.72	

* C.D. at 5% for variety, tiller age, and surface = ± 0.084; for leaf age = ± 0.14.

TABLE 7

VARIETY AND SURFACE EFFECTS UPON NUMBER OF EPIDERMAL CELLS (EXPERIMENT I)

VARIETY	SURFACE		MEAN	DIFFERENCE
	Lower	Upper		
Co 299	1440	1100	1270	
Co 213	1320	1200	1260	10
Co 290	1480	1220	1350	90
Co 421	1200	860	1030	320
Co S 76	1180	1100	1140	110
Co 453	1260	1020	1140	0
Co 312	1040	1240	1140	0
Rheora	1340	1280	1310	170
Co 356	1340	1380	1360	50
Co 385	1500	1340	1420	00
Co 205	1460	1180	1320	100
Co 313	1160	1000	1080	240
Mean	1310	1160	C.D. for varieties = ± 122.64	
Difference	150		C.D. for surface = ± 50.4	

could be divided into three classes on the basis of number of epidermal cells per unit area: class I, consisting of Rheora, Co 205, 290, 356, and 385 with a fairly high number of epidermal cells, ranging between 1310 and 1420 per unit area; class II, with a medium range of epidermal cell number of 1260–1270, characteristically noted in Co 213 and 299; and class III, consisting of Co 421, 313, Co S 76, Co 453, and Co 312 with the least number of epidermal cells, ranging between 1030 and 1140. The differences between the first and the third classes were distinctly significant. Among the different members of the same group the variations did not reach the level of significance in all cases.

In the second experiment the number of epidermal cells also varied significantly with variety, surface, and leaf age (table 2). The first and higher order interactions were also significant. Difference between the oldest and the youngest tiller was not significant at all (table 8); this was also the case with the varieties Co 421 and 205. The lower surface of the leaf, on the contrary, exhibited a significantly larger number of epidermal cells per unit area as compared with the upper surface. Increase in age of the leaf from the youngest to the second leaf again showed significant increase in the number of epidermal cells per unit area; subsequent advance in leaf age, however, did not cause significant variation.

TABLE 8*

TILLER AGE, SURFACE, VARIETY, AND LEAF AGE EFFECTS UPON NUMBER OF EPIDERMAL CELLS (EXPERIMENT II)

LEAF AGE	TILLER AGE		SURFACE		VARIETY		MEAN
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	1015	980	1010	985	950	1045	997.5
II	1095	1060	1180	975	1095	1060	1077.5
III	1000	1075	1115	960	1025	1050	1037.5
IV	1095	1030	1160	965	1010	1115	1062.5
Mean	1051.2	1036.2	1116.2	971.2	1020.0	1067.5	

* C.D. at 5% for variety, tiller age, and surface = ± 32.2; for leaf age = ± 45.64.

CHARACTERISTICS OF STOMATA

LENGTH.—The average length of stomata also differed characteristically. Main effects due to variety and surface and to variety × surface interaction were highly significant (table 1). The lower surface of the leaf on an average bore stomata with shorter length than the upper (table 9). Differences between surfaces were significant. Co 453 exhibited the maximum, Co 205 the minimum length of stomata. Based upon this character, the varieties could be classified as follows: class I, including Co 421, 313, Co S 76, and Co 453 with high stomatal length, ranging between 39.42 and 42.66 μ; class II, with medium length of stomata from 35.37 to 38.08 μ, including Co 385, 213, 299, 356, and 312; and class III, consisting of Co 205, Rheora, and Co 290 with an average stomatal length of 32.40–34.56 μ. One class as a whole differed from the other more characteristically than the varieties within a single class.

In experiment II all the main effects except that due to tiller age were highly significant. The first and higher order interactions were also significant (table 1). Differences between the oldest and the youngest tiller were not significant at all (table 10). The upper surface of the leaf showed significantly greater length of stomata than the lower. Of the two varieties, Co 421 exhibited the greater length of stomata. The youngest leaf showed the maximum and the second the least stomatal length, the differences between the two being significant. Increase in development from the third- to the fourth-leaf stage was, however, not significant in its effect on this character.

BREADTH.—Main effects due to variety and interaction due to variety and surface were both significant; differences due to surface were not marked (tables 1 and 11). The average stomatal breadth was maximum for Co 213 and minimum in Rheora. The difference between the

TABLE 9

VARIETY AND SURFACE EFFECTS UPON LENGTH OF STOMATA
(EXPERIMENT I)

VARIETY	SURFACE		MEAN	DIFFERENCE
	Lower	Upper		
Co 299	37.26	37.80	37.53	
Co 213	38.34	36.18	37.26	0.27
Co 290	29.16	39.96	34.56	2.70
Co 421	37.26	41.58	39.42	4.86
Co S 76	35.64	47.52	41.58	2.16
Co 453	40.50	44.82	42.66	1.08
Co 312	41.04	36.72	38.88	3.78
Rheora	32.40	35.10	33.75	5.13
Co 356	42.12	33.48	37.80	4.05
Co 385	33.48	37.26	35.37	2.43
Co 205	31.86	32.94	32.40	2.97
Co 313	41.04	37.80	39.42	6.98
Mean	36.6	38.4	C.D. for varieties = ±1.82	
Difference	1.8		C.D. for surface = ±0.52	

TABLE 10*

TILLER AGE, SURFACE, VARIETY, AND LEAF AGE EFFECTS UPON LENGTH OF STOMATA
(EXPERIMENT II)

LEAF AGE	TILLER AGE		SURFACE		VARIETY		MEAN
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	39.95	40.85	40.20	40.6	42.95	37.85	40.40
II	37.55	35.95	35.20	38.3	38.75	34.75	36.75
III	38.25	37.75	38.60	37.4	40.70	35.30	38.0
IV	38.15	38.65	38.05	38.7	41.50	35.30	38.40
Mean	38.47	38.30	38.01	38.70	40.97	35.80	

* C.D. at 5% for variety, tiller age, and surface = ±0.448; for leaf age = ±0.644.

two extremes was highly significant. Varieties could be classified as follows: class I, consisting of Co 356 and Co 213 with high stomatal cell width of 23.49–24.30 μ; class II, including Co 312, 299, 421, 290, and Co S 76, with medium stomatal breadth ranging between 20.25 and 21.60 μ; and class III, comprising Rheora, Co 453, 205, 313, and 385, with stomatal cell width of 17.01 to 19.71 μ.

TABLE 11

VARIETY AND SURFACE EFFECTS UPON BREADTH OF STOMATA
(EXPERIMENT I)

Variety	Surface		Mean	Difference
	Lower	Upper		
Co 299	20.52	21.60	21.06	
Co 213	22.68	25.92	24.30	3.24
Co 290	21.60	21.60	21.60	2.70
Co 421	21.06	21.06	21.06	0.54
Co S 76	23.76	19.44	21.60	0.54
Co 453	16.20	18.90	17.55	4.05
Co 312	21.06	19.44	20.25	2.70
Rheora	17.82	16.20	17.01	3.24
Co 356	24.30	22.68	23.49	0.48
Co 385	17.82	21.60	19.71	3.78
Co 205	20.52	16.20	18.36	1.35
Co 313	18.90	19.98	19.44	1.08
Mean	20.52	20.38	C.D. for varieties = ±1.344	
Difference	0.14		C.D. for surface = ±0.532	

As a group the varieties of class III differed significantly in stomatal breadth from the members of class I or II.

So far as stomatal breadth is concerned, data recorded in experiment II indicate that all main effects except that due to tiller age were significant (table 2). Differences in width of stomata on the oldest and the youngest tillers did not reach the level of significance (table 12). The lower surface of the leaf showed greater stomatal width than the upper. Co 205 showed greater average breadth of stomata than Co 421. Differences between the first and second leaf were not significant. Subsequent advance in age and development of leaf caused significant reduction in the width of the stomata.

NUMBER.—On stomatal number the main effects of variety and surface and of interaction due to variety and surface were again significant (table 1). On an average the stomatal number per unit area on the lower surface was distinctly higher than on the upper (table 13). The average values for different varieties showed the highest stomatal number in the case of Co 290, and the lowest in the case of Co 453; the difference between the two was highly significant. Again the varieties could be classified into: class I, consisting of Co 312, 313, and 290, with stomata ranging above 600 per unit area; class II, including Co 356, Rheora, 385, 299, and 213, with a range of 550–590 per unit area; and class III, containing Co 453, 205, 421, and Co S 76, having a range of 440–540 stomatal number per unit area.

In the second experiment the main effects due to tiller age and surface were

highly significant on stomatal number (table 2). First and higher order interactions were also significant in effect. Leaves on the oldest tiller showed significantly higher frequency of stomata per unit surface than leaves on the youngest tiller (table 14). The lower surface also showed a distinctly greater number of stomata than the upper. Varieties did not indicate any significant variation in stomatal frequency. Differences due to leaf development were also insignificant in so far as this character was concerned.

Stomatal index

Stomatal index calculated after Salisbury (**40**) significantly varied with the variety and surface (table 1). Variety × surface interaction was also significant. The lower surface of the leaves distinctly showed a higher stomatal index than the upper (table 15); the difference between the two was statistically significant.

TABLE 12*

Tiller age, surface, variety, and leaf age effects upon breadth of stomata (experiment II)

Leaf age	Tiller age		Surface		Variety		Mean
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	20.65	21.20	21.15	20.70	21.40	20.45	20.92
II	21.25	21.25	21.95	20.55	20.05	22.45	21.25
III	20.25	19.90	20.50	19.65	20.10	20.05	20.07
IV	19.45	18.45	19.75	18.15	18.15	19.75	18.95
Mean	20.40	20.20	20.83	19.76	19.90	20.60	.....

* C.D. at 5% for variety, tiller age, and surface = ± 0.308; for leaf age = ± 0.42.

TABLE 13

Surface and variety effects on stomatal number (experiment I)

Variety	Surface		Mean	Difference
	Lower	Upper		
Co 299	880	280	580	
Co 213	680	500	590	10
Co 290	800	460	630	40
Co 421	700	320	510	120
Co S 76	680	400	540	30
Co 453	520	360	440	100
Co 312	760	440	600	160
Rheora	780	360	570	30
Co 356	620	480	550	20
Co 385	680	460	570	70
Co 205	660	340	500	70
Co 313	800	420	610	110
Mean	713.3	401.6	C.D. for varieties = ± 104.72	
Difference	301.7		C.D. for surfaces = ± 43.12	

Co 313 with 35.2 stomatal index was the maximum and Co 205 with an index of 26.39 the minimum. The varieties could be classified into: class I, including Co 312 and 313 with stomatal indexes above 31.70; class II, consisting of Co 290, 213, Co S 76, and Co 421 with a range of 31.07–21.70; class III, including Co 205, 453, 385, 356, 299, and Rheora with stomatal index ranging between 26.39 and 29.37. As a class, members of class I differed significantly from those of class III. Variations among varieties belonging to one class were not significant at all.

On stomatal index, the main effects of tiller age and surface were highly significant (table 2). First-order interactions due to variety × surface, surface × tiller age, and surface × leaf age, and higher-order interactions were also significant. Leaves on the oldest tiller showed a higher stomatal index than on the youngest (table 16). Differences between the two

TABLE 14*

TILLER AGE, SURFACE, VARIETY, AND LEAF AGE EFFECTS UPON STOMATAL NUMBER (EXPERIMENT II)

Leaf age	Tiller age		Surface		Variety		Mean
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	535	485	620	400	485	535	510.0
II	575	480	675	380	555	500	527.5
III	505	470	700	335	530	505	517.5
IV	560	495	720	335	495	560	527.5
Mean	558.7	482.5	678.7	362.5	516.2	525.0	

* C.D. at 5% for variety, tiller age, and surface = ± 14.28; for leaf age = ± 19.96.

TABLE 15

VARIETY AND SURFACE EFFECTS UPON STOMATAL INDEX (EXPERIMENT I)

Variety	Surface		Mean	Difference
	Lower	Upper		
Co 299	37.4	20.24	28.82	
Co 213	33.92	29.38	31.65	2.83
Co 290	34.96	27.18	31.07	0.58
Co 421	36.38	27.02	31.70	0.63
Co S 76	36.34	26.66	31.50	0.20
Co 453	28.44	26.00	27.22	4.28
Co 312	41.70	25.98	33.84	6.62
Rheora	36.80	21.94	29.37	4.47
Co 356	31.14	25.62	28.38	0.99
Co 385	31.24	25.48	28.36	0.02
Co 205	30.62	22.16	26.39	1.97
Co 313	40.68	29.72	35.20	8.81
Mean	34.9	25.6	C.D. for varieties = ± 3.36	
Difference	9.3		C.D. for surface = 1.40	

varieties Co 421 and 205 were not significant. The youngest leaf showed a higher stomatal index than leaves of later stages of development. No significant difference was recorded between the second, third, and fourth leaves.

Discussion

A perusal of the data indicates the outstanding fact that the varieties under investigation could be classified into three major classes, namely, varieties with (*a*) low, (*b*) medium, and (*c*) high cell indexes. This is evident from figure 1, in which the actual values of different cell characters are plotted in increasing order of varieties (table 17). The relative position of the variety on the curve shows the general tendency of some varieties, viz., Co 205, 385, 453, and Rheora to exhibit lower values for at least 60% of the cell characters under study. Such varieties, therefore, belong to group I with low cell indexes. In contrast to this, there is another group exhibiting high cell index values, prominently exhibited by Co 213, 312, 421, and Co S 76. As a rule, in more than 60% of the characters they occupy a relatively higher position on the curve than others. In between these two groups there is the third class of intermediaries with occasional tendencies to approach either of the other groups. This class is represented by Co 290, 299, 356, and 313. Of these Co 313 notably exhibits 50% of cell index characters in favor of group I and 50% in favor of group III. Co 356 also tends to exhibit at least 40% of characters like varieties of group I and 40% like those of group III.

Stricter comparison, however, indicates that the variations in each of the cell characters do not always, and in all cases, follow any rigid system of classification or grouping of varieties. There are certain cell characters—for instance, breadth of the stomata and epidermal cells and the stomatal index—where the lower the values, the greater appears to be the tendency of the variety to align itself with group I. Similarly in group III with high cell indexes the stomatal index and the breadth of the epidermal cell are helpful in demarcating the four varieties Co 213, 312, 421, and Co S 76 from those of group I. The majority of these varieties exhibit high values for these two characters particularly. Within this group a tendency to exhibit a higher stomatal length and a greater length/breadth ratio of the stomata is also recorded.

TABLE 16*

TILLER AGE, SURFACE, VARIETY, AND LEAF AGE EFFECTS UPON STOMATAL INDEX (EXPERIMENT II)

LEAF AGE	TILLER AGE		SURFACE		VARIETY		MEAN
	Oldest	Youngest	Lower	Upper	Co 421	Co 205	
I	33.72	32.84	37.71	28.85	33.16	33.40	33.28
II	33.15	30.76	36.03	27.88	32.55	31.36	31.95
III	34.57	29.17	37.99	25.75	32.93	30.82	31.87
IV	32.83	30.85	38.02	25.66	32.97	31.71	31.84
Mean	33.55	30.90	37.93	27.03	32.65	32.82	

* C.D. at 5% for variety, tiller age, and surface = ± 1.176, for leaf age = ± 1.68.

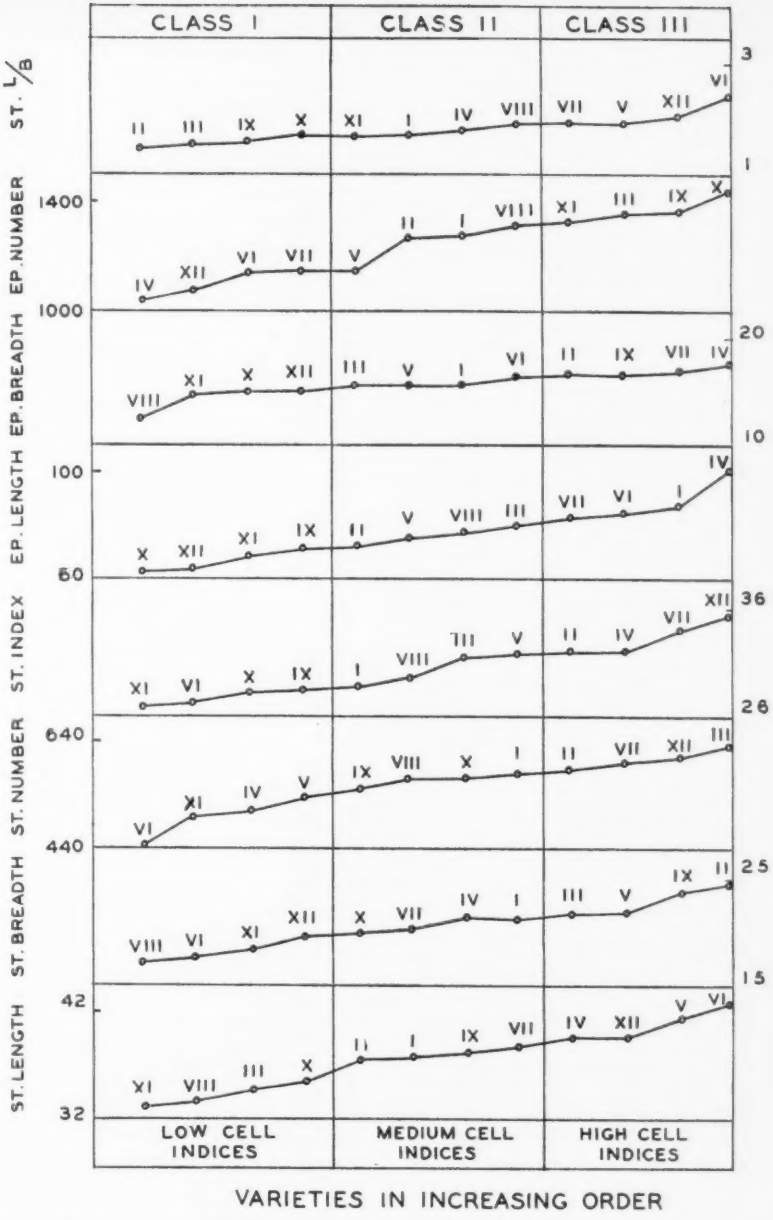

FIG. 1.—Cell characters of sugar-cane varieties: I, Co 299; II, 213; III, 290; IV, 421; V, Co S 76; VI, Co 453; VII, Co 312; VIII, Rheora; IX, Co 356; X, Co 385; XI, Co 205; and XII, Co 313.

Such variations in cell characters of varieties have also been recorded by earlier investigators for other crops. It is, however, difficult at this stage to ascertain to what extent such measurements of cell indexes would be of ecological significance in sugar cane. In other crops SALISBURY (**40**) has pointed out that stomatal frequency, particularly, has no adaptational significance in relation to certain physiological functions like transpiration and assimilation.

ly nondrought-resistant variety. The lower the indexes, the greater appears to be the drought-resistant capacity of the plant and vice versa. The fact that the variations are not sharp and significant among most of the varieties indicates that a great deal of caution is needed in taking these cell indexes as a measure of drought resistance. FRANCO (**10**) has pointed out that among closely related varieties of the same chromosomal number it is not possible to find any

TABLE 17

CHARACTERISTICS OF EPIDERMAL AND STOMATAL CELLS OF VARIETIES (EXPERIMENT I)

VARIETY	STOMATA					EPIDERMAL CELLS			CHROMOSOME NUMBER 2n
	Length	Breadth	Number	Index	L/B	Length	Breadth	Number	
Rheora	33.75	17.01	570	29.37	1.9	77.41	12.96	1310	82
Co 205	32.40	18.36	500	26.39	1.7	68.50	14.58	1320	112
Co 453	42.66	17.55	440	27.22	2.4	83.43	16.20	1140	.....
Co 312	38.88	20.25	600	33.84	1.9	82.54	16.74	1140	118
Co 421	39.42	21.06	510	31.70	1.8	99.36	17.28	1030	118
Co 299	37.53	21.06	580	28.82	1.7	87.40	15.39	1270	.....
Co 213	37.26	24.30	590	31.65	1.5	72.09	16.20	1260	118
Co 290	34.56	21.60	630	31.07	1.6	79.65	15.38	1350	.....
Co 356	37.80	23.49	550	28.38	1.6	71.55	16.20	1360	116
Co 385	35.37	19.71	570	28.36	1.6	63.4	14.85	1420	.....
Co 313	39.42	19.44	610	35.20	1.9	64.38	15.12	1080	118
Co S 76	41.58	21.60	540	31.50	1.9	75.6	15.39	1140	.....
C.D. 5%	1.82	1.34	104.7	3.36	.....	11.64	1.68	122.6	.....

Stomatal index, however, has been noted to be higher in the case of plants of drier habitats than in plants growing under relatively humid conditions. CARROLL and WELTON (**4**), on the other hand, found no significant difference among different species of turf grasses. The evidence recorded for sugar cane in the present instance, based upon the established drought-resistant behavior of at least the Rheora variety, points out that the dimensions of the stomata and of the epidermal cells to a certain extent may enable the investigator to segregate a drought-resistant form from a relative-

sharp difference in the size of the stomata. The same probably may be true of epidermal cells as well, though direct evidence is yet lacking. Observations of SAMARINA and KOLELISHVIBI (**41**) also indicate the possibility of assessing drought resistance in terms of a higher ratio of the length of palisade parenchyma to spongy parenchyma and of a large number of stomata in comparable areas. In the latter case abnormalities have been noted in certain species as well, thus making this character a less significant criterion of drought resistance in plants.

Measurements taken in the present experiments, when studied in relation to the chromosome numbers of different varieties, show that there is a tendency of certain cell characters to increase with the chromosome number, although this is not strictly the case in almost all the cell measurements recorded in this investigation (table 18). The relationship is particularly true in the case of breadth of the epidermal cells where this character increases with increase in the chromosome number. Except in Rheora with eighty-two chromosomes, other characters, such as length of the stomata, stomatal index, and length and breadth of epidermal cells, are also noted to increase with a rise in the chromosome number of the other three varieties—Co 205, 356, and 421. It is peculiarly true for variety Rheora with a chromosome number below 112 to show a somewhat different behavior when compared with other varieties with chromosome numbers above 112. In the case of stomatal number there appears to be some tendency to exhibit a fall with increase in chromosome number. Although this relationship is not very strict, some positive relationship is evident between chromosome number and certain cell characters —for instance, length and breadth of stomata, stomatal index, and length and breadth of epidermal cells; an inverse relationship is also visualized in the case of stomatal number. How far and in what manner the chromosome number—a fixed hereditary character of the variety —determines the variations in these external manifestations of cell size is very difficult to explain at present, in view of the scanty information available on this aspect of the problem of sugar-cane physiology.

TABLE 18

CHROMOSOME NUMBER AND CELL CHARACTERISTICS (EXPERIMENT I)

VARIETY	STOMATA					EPIDERMAL CELLS			CHROMOSOME NUMBER 2n
	Length	Breadth	Number	Index	L/B	Length	Breadth	Number	
Rheora	33.75	17.01	570	29.37	1.9	77.41	12.96	1310	82
Co 205	32.40	18.36	500	26.39	1.7	68.50	14.58	1320	112
Co 356	37.80	23.49	550	28.38	1.6	71.55	16.20	1366	116
Co 421	39.42	21.06	510	31.07	1.8	99.36	17.28	1030	118

KOLKUNOV (**22**) correlated cell size with certain growth characters—for instance, the period of maturity; varieties with smaller cells matured earlier than that with larger ones. These observations were, however, not confirmed by those of YAKUSHKIMA and VAVILOV (**58**), who pointed out no rigid connection between the size of the cells and the growth period, intensity of tillering, yield, or fungal attack. It is indeed difficult to visualize any superficial relationship between the dimensions of cells and the intensity of growth exhibited by any particular crop. As MAXIMOV (**29, 30**) has pointed out, internal characteristics of the protoplasm, its physiological functioning, and other physical and chemical properties would undoubtedly play more vital roles than do the mere superficial morphological and anatomical peculiarities of the plant. This is equally true for drought-resistance capacity as well.

Evidence collected on the effect of certain internal factors on the dimensions of various cell characters conclu-

sively point out that in sugar cane the effect of leaf surface, age, and development of leaf, and the relative maturity of tillers are of as great importance in determining cell size as the varietal factor itself. Of all these, the leaf surface characteristically brings about significant variations in six out of seven cell characters under investigation. This fact has been found by WESTERMEIER (54) to hold good in other species where the upper surface of the leaves with its stomata has been found to have a more decisive influence on the properties of the plant, particularly its developmental characteristics, than the lower surface. In the present work leaf age and age of the tillers show significant effects only in a few cases. The size of the stomata and breadth and number of epidermal cells varied significantly with the leaf age and varieties. Number of stomata, stomatal index, and length and breadth of epidermal cells, on the other hand, showed significant variations in response to both the age and the development of tillers.

A comparative survey of the responses reveals that the surface of the leaf from which samples are taken is a very important factor determining almost all cell-dimensional characters of the leaf. In all comparative studies, therefore, adequate allowance must be made for the variations due to surface and other developmental characters, such as the leaf age and tiller age. Comparative effects of all these factors in experiment II show that varietal responses on size of stomata and number and breadth of stomatal cells are likely to prove of greater utility in determining the intrinsic behavior of the varieties than are the other cell indexes under consideration. How far these and other characters can help in judging or in segregating drought-resistant types from relatively less drought-resistant strains will be discussed at a later stage when further information on other aspects of sugar-cane physiology have been presented.

Summary

1. This paper deals with the cell-size characteristics of certain sugar-cane varieties in relation to such internal factors as surface, leaf age, and tiller age. The varieties have been classified on the basis of such studies into three distinct groups, individual members of each of which show distinct similarities in various cell characters, thus: (*a*) *varieties with low cell indexes:* Co 205, 385, 453, and Rheora; (*b*) *varieties with medium cell indexes:* Co 290, 299, 356, and 313; and (*c*) *varieties with high cell indexes:* Co 213, 312, 421, and Co S 76. As a rule, varieties belonging to group I show significant differences in the majority of cell-index characters when compared with those of group III. Based upon the performance of Rheora, a drought-resistant variety, it is suggested that the lower the indexes, the greater appears to be the drought-resistant capacity of the variety.

2. Various internal factors appear to determine the size of cells. Of these, the chromosome number, the surface of leaf, the age of leaves, and the relative maturity of tillers are the most important. In all comparative studies of cell size these factors have therefore to be carefully scrutinized.

3. The effect of each of these factors has been discussed at length in relation to the work of other investigators, and suggestions have been made regarding the extent to which they might be helpful in studies on drought resistance in this crop.

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EMBRYOLOGICAL STUDIES OF TWO MEMBERS OF THE PODOSTEMACEAE

BASHEER AHMED RAZI

Introduction

Embryological studies on the members of the Podostemaceae have been pursued by several investigators, reviews of which were given by SCHÜRHOFF (9) and SCHNARF (8). WENT (10–14), CHIARUGI (1), and HAMMOND (2) have contributed toward the knowledge of the embryology in the family. MAGNUS (3) has traced the development of the embryo sac of *Lawia zeylanica* Tul., *Podostemon subulatus* Gardn., *Hydrobrium olivaceum* Tul., *Farmeria metzgerioides* Willis, and *Dicraea elongata* Tul. In the present study some aspects of the morphology of the plants and the embryology of *Lawia zeylanica* Tul. and *Griffithella hookeriana* Warming are presented.

Material and methods

L. zeylanica is an herb with frondose thalli attached to smooth rocks in rapidly flowing streams. The thallus is suborbicular or stellate and much branched with ribbon-like flabelliform lobes. Leaves are numerous and simple, occurring on the upper surface of the thallus. The flowers are solitary and terminal, situated at growing points on the margins of the thallus. The pedicels emerge from the terminal cupules located in the leaf axil. The pedicel and the ovary surface are covered with a pellucid cortical tissue which falls off after flowering.

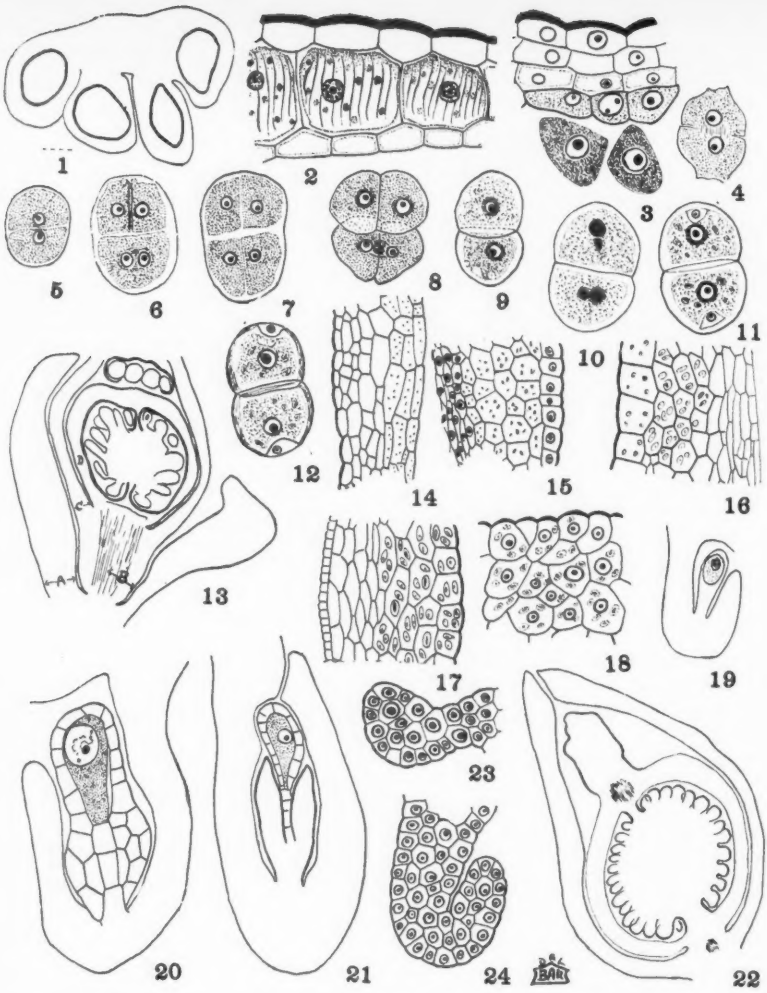
G. hookeriana is similar in habit to *L. zeylanica* and is attached to the substratum by means of stout secondary roots as is *Podostemon ceratophyllum* (2). The thick, lichenoid thallus bears secondary shoots at its margins which are broad at the base, becoming narrow at the tip. The apex of the secondary shoot bears a solitary hermaphroditic flower inclosed in a cupule which opens irregularly at maturity. Each flower has a pellucid perianth inclosing a pair of monadelphous stamens, a pair of linear staminodes, and an ovoid superior ovary, smooth on the outside and bearing two stigmata.

The materials used were collected at Koppa and Gersoppa, Mysore, South India. They were fixed in formalin–acetic-alcohol and the customary methods of infiltrating and imbedding followed. Sections cut 14μ in thickness were stained in Heidenhain's iron-alum haematoxylin with eosine as a counterstain.

Observations

MICROSPORANGIUM AND MALE GAMETOPHYTE.—The structure of the microsporangium and the development of the male gametophyte were studied in *Griffithella*. The anthers are quadrilocular (fig. 1), the wall being composed of three layers external to the tapetum (fig. 3). The epidermis is covered by a thick cuticle. The middle layer consists of rectangular uninucleate cells (fig. 3), and the endothecium develops fibrillar thickenings (fig. 2). The tapetum remains uninucleate and is of the secretory type.

The microspore mother cells (fig. 3) round off and undergo the two meiotic divisions (figs. 4–8) to form the tetrads of microspores. After quadripartition the four spores separate in twos, with the result that at maturity the pollen grains occur in pairs (figs. 9–12). Similar paired



FIGS. 1–24.—Magnification × 1800 unless otherwise stated. Figs. 1–12, microsporogenesis in *G. hookeriana*. Fig. 1, cross section of anther. × 160. Fig. 2, wall of old anther. Fig. 3, wall of young anther and microspore mother cells. Fig. 4, first division of microspore mother cell. Fig. 5, microspore mother cell after first division. Fig. 6, microspore mother cell in second division. Fig. 7, isobilateral tetrad of microspores. Fig. 8, separation of microspore tetrads in pairs. Fig. 9, paired microspores. Fig. 10, division of microspore to form generative and tube nuclei. Fig. 11, formation of tube cell and generative cell. Fig. 12, paired pollen grains at shedding stage; note starch grains and laterally thick exine. Figs. 13–21, ovary and ovule of *L. zeylanica*. Fig. 13, longisection of flower at megaspore mother-cell stage; note cupule (*A*), stalk (*B*), perianth (*C*), ovary (*D*), and one anther in cross section. × 80. Fig. 14, part of cupule at region marked *A* in fig. 13. × 400. Fig. 15, part of stalk at region marked *B* in fig. 13; here single epidermal layer; isodiametric starch-containing cells, and only three or four layers of central column are represented. × 400. Fig. 16, part of perianth at region marked *C* in fig. 13. × 800. Fig. 17, part of ovary wall at region marked *D* in fig. 13. × 800. Fig. 18, some cells of thick placenta. × 400. Fig. 19, young anatropous ovule with single archesporium. × 800. Fig. 20, young anatropous ovule with single archesporium; note row of three cells beneath archesporium, and beginning of inner integument. Fig. 21, longisection of ovule at megaspore mother-cell stage; note inner integument and micropyle. × 800. Figs. 22–24, ovary and ovule of *G. hookeriana*. Fig. 22, longisection of flower to show perianth and massive placenta bearing large number of ovules. × 80. Fig. 23, ovule primordium. × 560. Fig. 24, ovule primordium after curvature. × 560.

pollen grains have been recorded for species of *Podostemon*, *Dicraea*, and *Hydrobrium* by MAGNUS (3) and for species of *Mniopsis*, *Lophogyne*, and *Angolea* by WENT (11, 12).

Further development gives rise to pollen grains having a thick outer exine and a thin delicate intine. There is a large, deeply stained tube cell and a smaller lenticular generative cell (figs. 11, 12). Starch grains are distributed within the tube cell. The two generative cells occupy positions corresponding to the two poles of the double pollen grain.

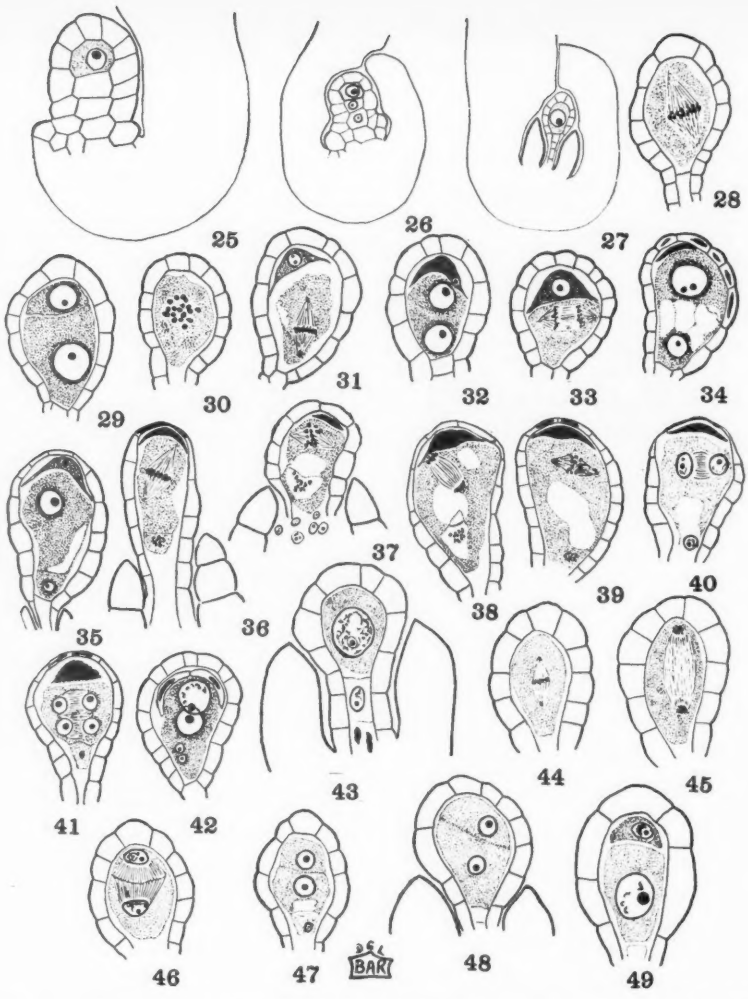
MEGASPORANGIUM AND FEMALE GAMETOPHYTE.—The ovary is superior and bilocular. A large number of anatropous ovules are borne on the swollen axile placenta, which is more fleshy in *Griffithella* (fig. 22) than in *Lawia* (fig. 13). The structures of the cupule, perianth, pedicel, ovary wall, and placenta are similar in both the species under study. In *Griffithella* the cupule as well as the thallus contains a large number of air spaces (fig. 67), as has also been observed by HAMMOND (2) for *Podostemon ceratophyllum*. In *Lawia* at the region of the point of attachment of the pedicel, the cupule shows a layer of epidermis and seven to ten layers of polygonal to elongated cells. The cells composing the innermost three to four layers have starch grains which are stained deeply by iodine potassium iodide (fig. 14). The flower stalk (fig. 15) shows two distinct zones—a central column of elongated cells with rich protoplasmic contents and large nuclei and a peripheral zone of three to four layers of isodiametric cells which are rich in starch grains. The perianth also shows the starchy and nonstarchy zones at the region of its attachment to the receptacle. The nonstarchy layer is innermost and comprises three or four layers of narrow, elongated cells (fig. 16). In the ovary wall (fig. 17) the outer epidermis incloses cells which are similar in structure and appearance to those comprising the nonstarchy layer of the perianth. The three or four layers between this zone and the inner epidermis are large and contain starch grains. The cells of the inner epidermis are larger than those of the outer. The placenta consists of irregularly arranged polygonal cells which have prominent nuclei and possess numerous starch grains (fig. 18).

In *Griffithella* a few xylem elements were noticed in sections of both the cupule and the thallus. Critical studies have not been made in *Lawia* to confirm the presence of vascular elements.

The ovule is anatropous and bitegmic, with the outer integument alone forming the micropyle (figs. 21, 26, 27, 65, 66, 68). As in the other members of the Podostemaceae so far investigated, the outer integument arises first and completely surrounds the nucellus even before the archesporium is differentiated (fig. 20). The inner integument develops later (fig. 26), and even at the megaspore mother cell stage it extends only as far as the basal portion of the megaspore mother cell (fig. 27).

The nucellus consists of an axial row of four or five cells covered by the nucellar epidermis (figs. 23, 24). The hypodermal cell enlarges to become the archesporial cell. It contains a prominent nucleus and dense cytoplasm (figs. 19, 20, 25). The cells below this are also elongated in the direction of the long axis of the ovule (fig. 20). At this stage the primordium of the inner integument is laid down near the base of the archesporium (figs. 20, 25, 26).

In a whole mount of the ovary of *Griffithella* cleared in lactophenol, an abnormal ovule showing a protrusion of the nucellus beyond the outer integument



FIGS. 25–49.—Magnification × 1800 unless otherwise stated. Figs. 25–27, ovary and ovule of *G. hookeriana*. Fig. 25, archesporium; note initial of outer integument. Fig. 26, longisection of ovule to show outer integument and micropyle at archesporium stage; note initials of inner integument. Fig. 27, longisection of ovule at megaspore mother-cell stage; note fully formed inner integument. × 540. Figs. 28–42, megaspore mother cell and its derivatives in *L. zeylanica*. Fig. 28, megaspore mother cell in division. Fig. 29, dyad; note smaller size of micropylar dyad cell. Fig. 30, metaphase plate showing eighteen chromosomes during division of megaspore mother cell. Fig. 31, chalazal dyad cell dividing. Fig. 32, two-nucleate embryo sac with degenerating micropylar dyad cell. Fig. 33, chalazal dyad cell dividing transversely. Fig. 34, two-nucleate embryo sac with vacuoles between two nuclei; note small size of chalazal nucleus. Fig. 35, same; chalazal nucleus cut off by membrane. Fig. 36, same, nuclei dividing. Fig. 37, same; note inner integument and free nuclei of pseudo embryo sac just below embryo sac. Fig. 38, same; note vacuole between dividing nuclei. Fig. 39, nuclei of two-nucleate embryo sac dividing. Fig. 40, first division of micropylar nucleus. Fig. 41, four-nucleate embryo sac; note persistent spindles and degenerating antipodal cell. Fig. 42, four-nucleate embryo sac with two large nuclei at micropylar end and two small ones at chalazal end; note two cells in crescentic upper dyad cap. Figs. 43–49, megaspore mother cell and its derivatives in *G. hookeriana*. Fig. 43, megaspore mother cell and axial row, with fully formed inner integument. Fig. 44, megaspore mother cell in metaphase. Fig. 45, same in telophase. Fig. 46, same in late telophase. Fig. 47, dyad. Fig. 48, dyad with small micropylar cell. Fig. 49, uninucleate embryo sac with degenerating dyad cap.

was seen (fig. 63). This is similar to a case reported in *Podostemon ceratophyllum* (**2**).

In both *Griffithella* and *Lawia* the archesporium functions directly as the megaspore mother cell. It is large in size, oval in shape, has a conspicuous nucleus with dense cytoplasm (fig. 43), and divides meiotically (figs. 44–46). The inner integument at this stage is two cells thick (fig. 27). The axial row of cells beneath the megaspore mother cell elongates as development of the ovule proceeds (figs. 27, 43).

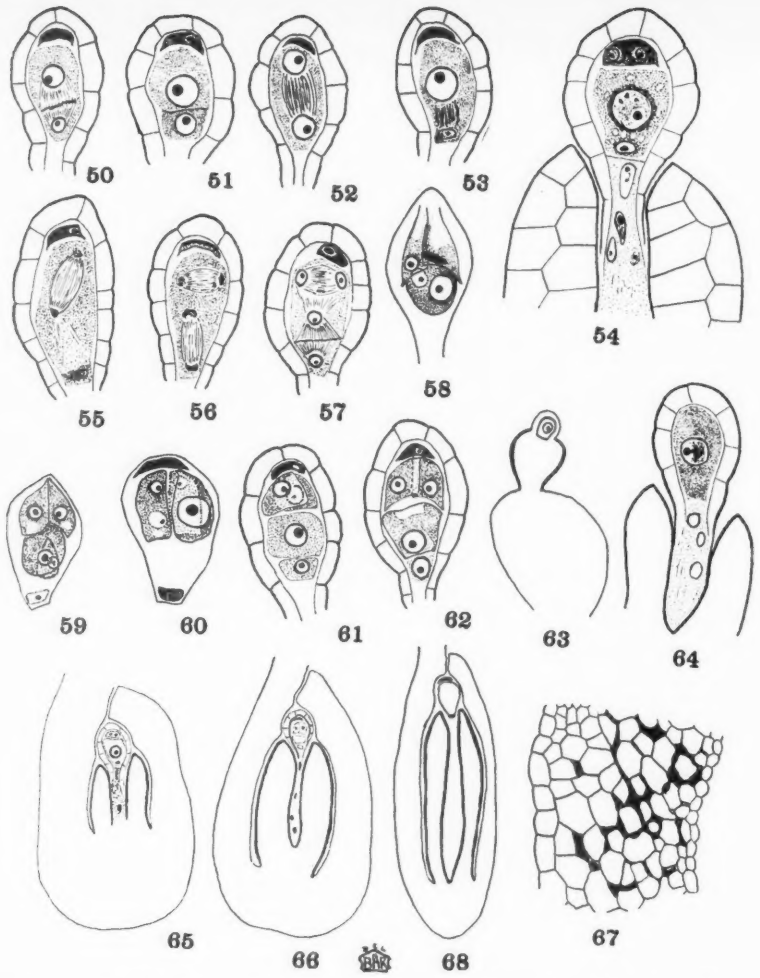
During the first division of the megaspore mother cell in *L. zeylanica* (figs. 28, 29) the metaphase count shows eighteen chromosomes (fig. 30). This is in contrast to the number reported for the same species by MAGNUS (**3**), who counted twenty chromosomes. CHIARUGI (**1**) recorded twenty haploid chromosomes for *Weddellina squamulosa*.

In *Lawia* the micropylar dyad cell degenerates, and the chalazal enlarges to form the embryo sac. The micropylar dyad cell may sometimes undergo an anticlinal division resulting in two laterally disposed cells (fig. 42). The nucleus of the chalazal dyad cell divides (figs. 31–33) to form two daughter nuclei. The lower nucleus degenerates soon after it becomes separated from the upper by the formation of a central vacuole (fig. 34). Sometimes the division is followed by the formation of a septum differentiating an antipodal cell (fig. 35). The latter either degenerates or undergoes an irregular mitosis (figs. 36–39). The micropylar nucleus meanwhile divides (figs. 36–40) twice, resulting in the micropylar quartet (fig. 41). Sometimes, as an abnormal variation, the four-nucleate embryo sac results from division of both the micropylar and the chalazal nuclei (fig. 42). At this stage the nucellar epidermis becomes crushed and cannot be recognized.

In *Griffithella* the micropylar dyad cell is smaller than the chalazal (figs. 47–49) and degenerates very early (fig. 49). The chalazal dyad cell divides once, and during this division a wall may (figs. 50, 51) or may not (figs. 52, 53) be laid down between the two daughter nuclei. When the wall is laid down, a chalazal or antipodal cell is formed (fig. 51) as in *Lawia*. Of the two nuclei, the chalazal one degenerates soon after the division is completed (fig. 53) and does not divide as in *Lawia*. The micropylar nucleus divides with its spindle disposed in the long axis of the ovule (fig. 55). The second division (fig. 56) follows so quickly that the spindles of the two nuclear divisions can frequently be seen at the same time (fig. 57).

EMBRYO SAC.—The mature embryo sac of *L. zeylanica* consists of an egg apparatus and an upper polar nucleus (fig. 58). Occasionally the antipodal cell or nucleus also persists (figs. 59, 60). This is in agreement with the observations on most of the other members of the Podostemaceae in which the embryo sac is five-nucleate with the complete elimination of the lower polar nucleus (**4, 5, 7**). The egg apparatus consists of the two synergids and a large egg (figs. 58–60). The egg cell is usually larger than the synergids, but sometimes all the three cells may be of the same size (fig. 60). The synergids normally show the typical basal vacuolation, though sometimes they are egglike with apical vacuolation (fig. 58).

During development of the embryo sac in *G. hookeriana* the micropylar nucleus of the chalazal dyad cell divides twice to form a four-nucleate sac. The complements of the chalazal quartet are lacking. Of the micropylar quartet, the two nuclei of the micropylar pair form



FIGS. 50–68.—Magnification × 1800 unless otherwise stated. Figs. 50–57, *G. hookeriana*. Fig. 50, formation of two-nucleate embryo sac; note small chalazal nucleus and phragmoplast. Fig. 51, two-nucleate embryo sac; note antipodal cell. Fig. 52, formation of two-nucleate embryo sac. Fig. 53, same, in late telophase; note small chalazal nucleus. Fig. 54, longisection of ovule at two-nucleate embryo-sac stage to show inner integument and pseudo embryo sac. × 540. Fig. 55, first division of micropylar nucleus; note degenerating antipodal cell. Fig. 56, second division of same; note two spindles at right angles to each other. Fig. 57, same; note persistent spindle between two divisions and phragmoplast in equator of lower spindle. Figs. 58–60, embryo sac in *L. zeylanica*. Fig. 58, mature embryo sac; note egglike synergids. Fig. 59, same, with degenerating antipodal cell. Fig. 60, same; note equal sizes of synergids and egg. Figs. 61–62, embryo sac in *G. hookeriana*. Fig. 61, four-celled embryo sac. Fig. 62, mature embryo sac; note persistent dyad cap. Figs. 63–67, pseudo embryo sac in *G. hookeriana*. Fig. 63, abnormal ovule showing a protrusion of nucellus from outer integument. Fig. 64, pseudo embryo sac at megaspore mother-cell stage. Fig. 65, two-nucleate embryo sac and pseudo embryo sac; note smaller chalazal nucleus and elongated nuclei in pseudo embryo sac. Fig. 66, longisection of ovule at mature embryo-sac stage to show micropyle, integuments, and elongated narrow pseudo embryo sac. × 540. Fig. 67, transverse section of thallose plant body with air spaces. × 100. Fig. 68, longisection of ovule of *L. zeylanica* at mature embryo-sac stage to show micropyle, inner integument, embryo sac, the pseudo embryo sac. × 540.

the synergids. The egg and the polar cell are formed by the lower pair. This polar cell is unique and, as far as the writer knows, has not been reported in any of the flowering plants so far investigated (6). The wall of the polar cell later on disappears to give a free polar nucleus (fig. 62). Unlike *Lawia*, the egg apparatus and the polar nucleus occupy the entire space within the embryo sac (figs. 61, 62).

PSEUDO EMBRYO SAC.—The pseudo embryo sac is a characteristic feature of all the Podostemaceae so far investigated (2, 3, 10). According to HAMMOND (2, p. 33), "as the ovule continues to develop, the cells of the nucellus near the base of the inner integument fail to divide rapidly enough to keep pace with the elongation of the integuments, and these cells are consequently drawn out into shred-like structures, later giving a cavity, the 'pseudo embryo-sac,' surrounded by the inner integument, and into which, according to Magnus and others, in all the species studied by them, the developing embryo grows." The present work on *L. zeylanica* tends to support HAMMOND's findings. In *G. hookeriana* the maximum development of the pseudo embryo sac is attained at the two-nucleate embryo-sac stage and gradually narrows down as the embryo sac matures. This is in contrast to the condition found in *Lawia*, where the pseudo embryo sac remains conspicuously large up to the mature embryo-sac stage (fig. 68). Observations indicate that at the two-nucleate embryo-sac stage the inner integument of *Griffithella* is composed of two layers of cells. As the embryo sac matures, the inner integument becomes massive and multilayered. The narrowing of the pseudo embryo sac may be due to the inward growth of the inner integument. During early stages the pseudo embryo sac shows three free nuclei (fig. 64) formed as a consequence of the breakdown of the axial row of cells. As the pseudo embryo sac enlarges, the contiguous cells of the nucellar epidermis break down, and their nuclei migrate into the pseudo embryo sac. Consequently, the pseudo embryo sac contains several nuclei (figs. 54, 66), many of them showing signs of elongation and degeneration. Thus, the pseudo embryo sac of *G. hookeriana* differs from that of *L. zeylanica* and *Podostemon ceratophyllum*.

Summary

1. This paper deals with some aspects of the morphology and gametogenesis of *Lawia zeylanica* and *Griffithella hookeriana*, members of the Podostemaceae. Some of the structural details of the two plants are described.

2. Microsporogenesis was investigated in *Griffithella*. The anthers are quadrilocular, inclosing paired pollen grains. At the shedding stage there is a lenticular generative cell and a large tube cell in each pollen grain.

3. Development of the female gametophyte was studied in both *Lawia* and *Griffithella*. The ovules are anatropous and bitegmic. The archesporial cell functions directly as the megaspore mother cell. The embryo-sac development corresponds to a reduced *Allium* type, in which there is reduction at the chalazal end, and consequently a complete elimination of the lower polar nucleus.

4. The mature embryo sac of *L. zeylanica* is usually four- or rarely five-nucleate, but the chalazal nucleus may soon degenerate and disappear, so that only four nuclei are seen in the older stages. Sometimes the chalazal nucleus may divide to give a six-nucleate embryo sac.

5. In *G. hookeriana* the mature embryo sac is four-nucleate because of early degeneration of the chalazal nucleus. A

polar cell is formed during the development of the embryo sac. This may be an unusual feature among angiosperms.

6. A prominent multinucleate pseudo embryo sac is seen in both the species, resulting from the disintegration of the axial row of cells.

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CURRENT LITERATURE

Entwicklungs- und Bewegungsphysiologie der Pflanze. By ERWIN BÜNNING. Berlin, Göttingen, Heidelberg: Springer Verlag, 1948. Pp. xii+464+404 figs. M. 29.70 (approx. $9.00.)

In 1939 the Springer Verlag began the publishing of a "Lehrbuch der Pflanzenphysiologie," starting with BÜNNING's *Die Physiologie des Wachstums und der Bewegungen*, which constituted the second volume of the whole work as planned. Volume I, *Die Physiologie des Stoffwechsels*, and Volume III, *Die Physiologie der Entwicklung*, written by K. MOTHES and F. VON WETTSTEIN, respectively, were expected to appear in 1939 and 1940. World War II upset the whole plan, and now, 10 years later, MOTHES is a prisoner of war in Russia and VON WETTSTEIN is dead.

The present book, appearing as Volumes II and III of "Lehrbuch der Pflanzenphysiologie," constitutes a second edition of the above-mentioned work by BÜNNING into which the physiology of development has been incorporated. It consists of six parts. The main content of Part I is a survey of the role played by the individual cell constituents in development. Part II deals with dormancy and the causes of changes of activity. The general physiology of growth and nuclear division is treated in Part III. The internal factors in differentiation (among others polarity and correlation phenomena) are described and analyzed in Part IV. The mechanisms of movements, other than growth movements, are treated in Part V. The author points out the interesting similarity between the opening movement of the fern sporangium and the process by which the *Utricularia* bladder becomes ready for catching. These two types of movement are grouped together as "movements caused by negative wall pressures," the sporangium with passive, *Utricularia* with active, removal of water.

The usual groups of movements—tropisms (photo-, geo-, etc.), nastic movements, etc.—are not treated in sequence but are dealt with partly in Part V and partly in Part VI, the latter headed "The Action of External Stimuli on Movement and Development." Each chapter of this section deals with the various responses which may result from exposure of a plant to a certain external factor. For instance, in the chapter "Influence of Visible Light" will be found: light-growth reactions, growth movements caused by light, phototaxis, light-turgor movements of guard cells, etiolation, and photoperiodism. The responses of the plant are classified according to the nature of the external factor which causes them, such as light, temperature, seasonal variations, gravity, etc. This ecological basis for classifying the physiological reactions has, in fact, been used previously. Since, however, the external factors are stimuli, the effect of which depends on the internal organization of cells and tissues, the nature of the response is now generally considered the natural, physiological basis for classification of plant responses. BÜNNING, of course, maintains the concept of stimuli. His motivation for returning to the "old" classification of plant responses is the view that each factor is first perceived by the plant by a common mechanism, whatever the final reaction may be. For instance, the primary action of light has been shown in many cases to be an absorption in carotenoids or in chlorophyll. BÜNNING's own research has contributed new information in this field. He attempts to find a common mechanism for all physiological stimulations and shows that in many cases the "all-or-none" principle determines the reaction of the individual cell, i.e., either the influence is so strong that it completely breaks down some labile system in the cell (the reaction being accompanied by electrical currents of action), or (if the influence is below a certain threshold value) nothing happens at all. A quantitative relationship between reaction and intensity of stimulus may then be explained by assuming that the number of reacting cells increases with increasing intensity of the stimulus.

His ideas of the relationship between photoperiodic responses and endogenous, rhythmical leaf movements are set forth very clearly. The latter movements are taken as an indication that the internal conditions in the plant vary rhythmically between a "day phase" and a "night phase." The response to light is quite different in these two phases.

The book is not so exquisitely manufactured as the first edition, but even on the somewhat lower grade of paper most of the photographs come out remarkably clear. Exceptions are figures 189 and 266. The reviewer welcomes the substitution of GOEBEL's drawing of the *Utricularia* bladder (fig. 218) for LLOYD's in the first edition. The citations of original papers are fewer than in the first edition (only the more recent ones are cited), but the references to reviews and monographs still enable the reader to find the original literature.

The book is not an elementary text. A knowledge of elementary general botany as presented in introductory textbooks is presupposed. Admitted this, the advanced student, the teacher of plant physiology, and the research worker will feel stimulated by reading and using this highly interesting book which has so much new information and presents many of the well-known facts from original viewpoints. For the person who especially wants a survey of growth and development, it offers much more than any other existing modern textbook.—POUL LARSEN.

Photosynthesis in Plants: A Monograph of the American Society of Plant Physiologists. Edited by JAMES FRANCK and WALTER E. LOOMIS. Ames, Iowa: Iowa State College Press, 1949. Pp. x+500+129 figs. $7.00.

The more recent contributions to our knowledge of photosynthesis in plants are presented and discussed in twenty-two essays by the thirty-three authors of this book. The symposium form has the advantage of presenting authoritative, firsthand information as closely up to date as technically possible. Its disadvantage is that the integration of the material as a whole is in general left to the reader. In the present case, however, the introduction by LOOMIS brings together under general views the main results of the subsequent articles.

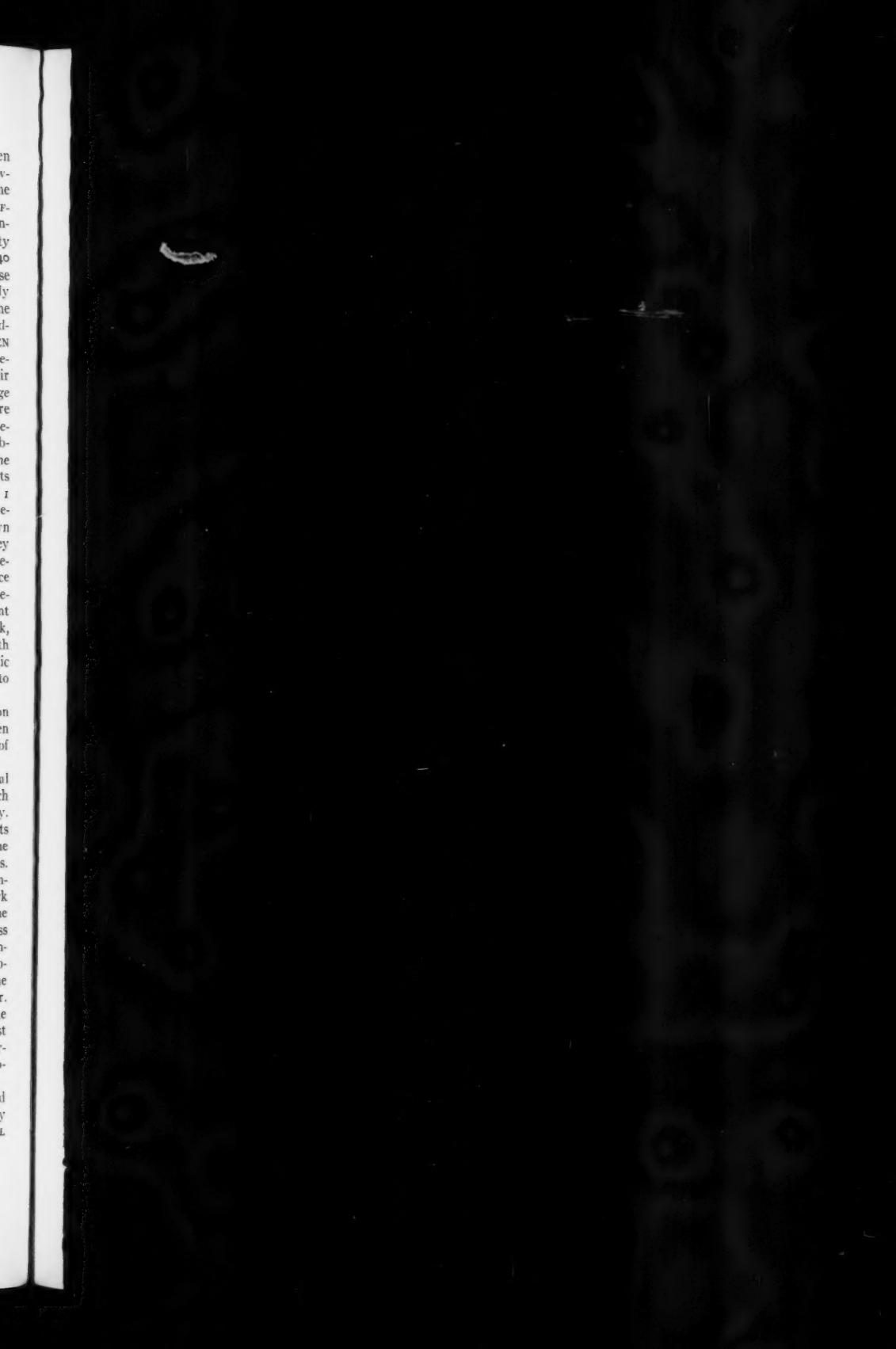
The contribution by THOMAS and HILL on photosynthesis under field conditions will be of interest to ecologists. The two authors found an increase in the rate of photosynthesis with an increasing CO_2 concentration up to ten times the normal one. Leaves of plants exposed to 0.3% CO_2 for 2 weeks, however, began to develop necrotic areas. The highest CO_2 tension at which plants may be grown undamaged for a whole life-cycle is still unknown. New data on the conditions for diffusion through multiperforate septa and for the interference between stomata are given by VERDUIN.

Chloroplasts and their pigments are dealt with in five articles. The existence of grana has been verified by use of the electron microscope. Fifty or more chloroplast pigments are recognized, most of them being carotenoids. Evidence is accumulating that chlorophyll *a* is actually formed from protochlorophyll (by a reduction). Four articles deal with the question, so important thermodynamically, of the quantum requirement: How many light quanta are required for the reduction of one mol of CO_2 in photosynthesis? The four present contributions are all in favor of a requirement of eight to twelve quanta and therefore in striking disagreement with the results obtained by WARBURG and his co-workers, whose renewed claim of the sufficiency of only four quanta is discussed in detail and rejected, mainly on the basis of a criticism of WARBURG'S technique.

New methods for the study of details in the mechanism of photosynthesis have been applied. One is the use of isolated chloroplasts; another is measurement of fluorescence of chlorophyll. Fluorescence seems to be inversely related to the rate of photosynthesis. The views on this point, however, are not unanimous. In connection with fluorescence measurements FRANCK concludes that certain intermediates in the process of photosynthesis may act as narcotics or poisons reducing the photosynthetic rate.

The tracer methods, which yielded such clear-cut results in proving that the oxygen evolved in photosynthesis originates from water, have been used extensively. The use of labeled carbon, however, has not given an equally clear picture of the nature of organic intermediates in the process. GAFFRON and his group have been able to isolate two intermediates which contain most of the radioactivity of the $C^{14}O_2$ which *Scenedesmus* has taken up in 40 seconds. Extensive experiments to identify these substances have so far been unsuccessful. The only positive, chemical characteristic is that some of the radioactivity is present in carboxyl groups. This finding is in accordance with the earlier work by RUBEN *et al.* In complete disagreement with GAFFRON'S results is the claim by CALVIN and BENSON and their group that the radioactivity is found in a large number of well-known substances, some of which are intermediates in respiration (photosynthesis as a reversed respiration process). The discrepancy is probably due to differences in the techniques used by the two groups. CALVIN'S group exposes the plants (*Chlorella*, *Scenedesmus*, barley) to labeled CO_2 for 1 or 5 minutes in the dark, immediately after a pre-illumination (several minutes) which has been shown to increase greatly the "dark fixation" of CO_2. They assume that the photochemical step in photosynthesis (formation of a reducing agent) has taken place during the pre-illumination period and that the reduction of CO_2 takes place during the subsequent dark period. When CO_2 is assimilated in the dark, there is the possibility, however, that it takes a path quite different from that followed in photosynthetic assimilation. The "dark fixation" may be similar to the fixation of CO_2 by heterotrophic organisms.

The final article by VAN NIEL gives a comparison between the processes of photosynthesis in green plants and in various bacteria and a discussion of photosynthesis on a broad biochemical basis.

Many of the contributions contain a good general discussion of their subject. Others appear as research reports. A few suffer from the lack of a summary. Comparatively few reviews of the ecological aspects of photosynthesis are given. The main part of the book is devoted to the mechanism of the process. The "monograph" thereby becomes somewhat unbalanced. In spite of the enormous amount of work which has been done to reveal the details of the mechanism, this is still obscure. Although progress has been made toward the separation of photosynthesis from organized elements, the living protoplasm seems still to be indispensable for linking the photochemical step and the dark reactions together. The statement in the prospectus, advertising the book, that plant photosynthesis is "the world's most important chemical process" is therefore an oversimplification. Photosynthesis in plants is a physiological process.

The work as a whole can be highly recommended as a survey of recent progress in the field, especially in the study of the mechanism of the process.—POUL LARSEN.